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(54) Title: HYDROLASE ENZYMES AND THEIR USE IN KINETIC RESOLUTION

SEQ ID NO.2 BD021

Val Ser Ile Arg Leu Arg Leu Leu Asn Trp Phe Leu Asn Thr Phe Glu Lys Pro Lys Leu Ala Ala Ala Lys Thr Pro  
Asp Asp Leu Arg Lys Ser Phe Glu Leu Lys Ala Arg Phe Leu Phe Pro Ala Pro Arg Lys Thr Arg Phe Ser His  
Asp Val Leu Gln Ser Gly Ile Gly Ser Val Asn Ala Gln Trp Ala Lys Ser Lys Ser Ala Ser Asp Asp Arg Val Ile  
Leu Tyr Phe His Gly Gly Gly Tyr Val Phe Gly Ser Pro Lys Thr His Arg Ala Met Leu Ala Arg Leu Ser Ala Met  
Thr Gly Leu Ser Ala Cys Leu Pro Asp Tyr Arg Leu Ala Pro Glu His Pro Phe Pro Ala Ala Ile Glu Asp Ala Val  
Leu Ser Tyr Lys Cys Leu Leu Glu Arg Ala Ile Glu Pro Gln Asn Ile Ile Leu Gly Gly Asp Ser Ala Gly Gly Gly  
Leu Val Leu Ala Leu Leu Ala Glu Ile Lys Ala Gln Ser Leu Pro Lys Pro Ala Gly Val Phe Ala Leu Ser Pro Leu  
Val Asp Leu Ser Phe Ser Gly Leu Ser Phe Ser Lys Asn Ala Gln Thr Asp Val Met Leu Pro Ala Ser Arg Ala Ala  
Asp Met Ala Thr Leu Tyr Leu Asp Gly Ala Asp Ala Asp Asp Pro Arg Ala Ser Pro Leu Gln Ala Asp Phe Ser  
Gly Met Pro Pro Val Phe Leu Thr Ala Ser Asp Ser Glu Ile Leu Leu Asp Asp Cys Leu Arg Met Ala Asp His Leu  
Arg Ala Gln Gly Val Val Val Thr Asp Arg Ile Val Glu Asn His Pro His Val Trp His Ile Phe Gln Arg Leu Leu  
Pro Glu Ala Asp Gln Gly Leu Arg Ala Ile Ala Ala Trp Ile Lys Pro Leu Leu Ser Gly Ser Asn Glu Ser

(57) Abstract: The invention relates to hydrolases and to polynucleotides encoding the hydrolases. In addition, the invention relates to the use of these hydrolase enzymes in kinetic resolution.

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## HYDROLASE ENZYMES AND THEIR USE IN KINETIC RESOLUTION

### CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims the benefit of U.S. Provisional Application Serial No. 60/250,976, filed on December 1, 2000.

### BACKGROUND OF THE INVENTION

#### Field of the Invention

- 0 This invention relates generally to enzymes, particularly those that have hydrolase activity. The invention also relates to the use of hydrolase enzymes for kinetic resolution.

#### Background of the Invention

- Hydrolases are enzymes that catalyze a hydrolysis reaction. Thus, hydrolases act to break down a compound (*i.e.*, the substrate) by cleaving a covalent bond in the compound and inserting a water molecule across the bond. The general class of hydrolase enzymes  
5 includes those that act on ester bonds, on carbon-nitrogen bonds, on peptide bonds, on glycoside bonds, on ether bonds, and on acid anhydrides, among others.

- The esterases and lipases are hydrolase enzymes that catalyze the hydrolysis of esters to organic acids (fatty acids in the case of lipases) and alcohols. Many esterases and lipases are known, and they have been discovered in a broad variety of organisms, including  
0 bacteria, yeast and higher animals and plants. The major industrial applications for lipases include: the detergent industry, where they are employed to decompose fatty materials in laundry stains into easily removable hydrophilic substances; in waste systems; in the pharmaceutical industry, where they are used as digestive aids; and in the food and beverage industry, where they are used in the manufacture of cheese, the ripening and flavoring of  
5 cheese, as antistaling agents for bakery products, and in the production of margarine and other spreads with natural butter flavors.

More recently, hydrolases, particularly esterases and lipases, have found widespread use in chemical synthesis (Bornscheuer and Kazlauskas, *Hydrolases in Organic Synthesis - Regio- and Stereoselective Biotransformations*, Wiley-VCH: Weinheim, 1999; Faber, *Biotransformations in Organic Chemistry*, 3 ed.; Springer: Berlin, 1997; Drauz and Waldmann, *Enzyme Catalysis in Organic Synthesis*; VCH: Weinheim, 1995; Vol. 1 & 2; Wong and Whitesides, *Enzymes in Synthetic Organic Chemistry*, Pergamon Press: Oxford, 1994). Because these enzymes often exhibit exquisite stereospecificity, one enantiomeric substrate will react at a dramatically faster rate than the other. This selective reaction of one enantiomer of a racemic starting material over the other is termed "kinetic resolution," and permits the isolation of the starting material, product, or both in enantiomerically enriched form. Chen *et al.*, *J. Am. Chem. Soc.* 104: 7294 (1982), describes a method for the quantitative analysis of enzymatic kinetic resolution of enantiomers.

Lipases and esterases have been used for the kinetic resolution of over 1,000 alcohols, carboxylic acids, and lactones by means of hydrolysis or transesterification, and several biotransformations involving the use of lipases are already performed on an industrial scale. Despite the widespread success of enzymatic resolution technology, however, a number of important synthetic building blocks remain difficult to resolve by this method. Kazlauskas *et al.*, *J. Org. Chem.*, 56: 2656 (1991), teaches that the most efficiently resolved substrates are those having substituents which differ significantly in size. By contrast, currently available enzymes exhibit poor enantioselectivity toward substrates having substituents that are similar in size. There thus remains a need in the art for new hydrolase enzymes, particularly those that exhibit high enantioselectivity toward substrates that are currently difficult to resolve.

## BRIEF SUMMARY OF THE INVENTION

The invention provides polypeptides with hydrolase activity. The hydrolase enzymes of the invention are capable of resolving enantiomeric mixtures of alcohol or ester substrates, including substrates that heretofore have been difficult to resolve. The invention also  
5 provides polynucleotides encoding polypeptides with hydrolase activity.

In a first aspect, therefore, the invention provides an isolated nucleic acid having a sequence as set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13 (hereinafter "Group A nucleic acid sequences"), and variants thereof having at least 50% sequence identity to a sequence as set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13, and encoding polypeptides having hydrolase  
10 activity. The invention also provides nucleic acid sequences substantially identical to or complementary to the nucleic acid sequences set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13.

Another aspect of the invention is an isolated nucleic acid including at least 10 consecutive bases of a sequence as set forth in Group A nucleic acid sequences, sequences substantially identical thereto, and sequences complementary thereto.

5 In yet another aspect, the invention provides an isolated nucleic acid encoding a polypeptide having a sequence as set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, or 14, and variants thereof encoding a polypeptide having at least 50% sequence identity to a sequence as set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, or 14, and having hydrolase activity.

Another aspect of the invention is an isolated nucleic acid encoding a polypeptide  
10 having a sequence as set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, or 14 (hereinafter referred to as "Group B amino acid sequences") or a sequence substantially identical thereto, or a functional fragment thereof.

Another aspect of the invention is an isolated nucleic acid encoding a polypeptide having at least 10 consecutive amino acids of a sequence as set forth in Group B amino acid  
5 sequences, and sequences substantially identical thereto.

In yet another aspect, the invention provides a purified polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. In various embodiments, the purified polypeptide has a sequence with at least 50% sequence

identity to a sequence as set forth in Group B amino acid sequences, and has hydrolase activity.

Another aspect of the invention is an isolated or purified antibody that specifically binds to a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is an isolated or purified antibody or binding fragment thereof, which specifically binds to a polypeptide having at least 10 consecutive amino acids of one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is a method of making a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid.

Another aspect of the invention is a method of making a polypeptide having at least 10 amino acids of a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The method includes introducing a nucleic acid encoding the polypeptide into a host cell, wherein the nucleic acid is operably linked to a promoter, and culturing the host cell under conditions that allow expression of the nucleic acid, thereby producing the polypeptide.

Another aspect of the invention is a method of generating a variant including obtaining a nucleic acid having a sequence as set forth in Group A nucleic acid sequences, sequences substantially identical thereto, sequences complementary to the sequences of Group A nucleic acid sequences, fragments comprising at least 30 consecutive nucleotides of the foregoing sequences, and changing one or more nucleotides in the sequence to another nucleotide, deleting one or more nucleotides in the sequence, or adding one or more nucleotides to the sequence.

Another aspect of the invention is a computer readable medium having stored thereon a sequence as set forth in Group A nucleic acid sequences, and sequences substantially

identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is a computer system including a processor and a data storage device wherein the data storage device has stored thereon a sequence as set forth in  
5 Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is a method for comparing a first sequence to a reference sequence wherein the first sequence is a nucleic acid having a sequence as set forth  
10 in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide code of Group B amino acid sequences, and sequences substantially identical thereto. The method includes reading the first sequence and the reference sequence through use of a computer program which compares sequences; and determining differences between the first sequence and the reference sequence with the computer program.

Another aspect of the invention is a method for identifying a feature in a sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide having a sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, including reading the sequence through the use of a computer program which identifies features in sequences; and identifying features in the sequence with  
15 the computer program.

Another aspect of the invention is an assay for identifying fragments or variants of Group B amino acid sequences, and sequences substantially identical thereto, which retain the enzymatic function of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. The assay includes contacting the polypeptide of Group B  
20 amino acid sequences, sequences substantially identical thereto, or polypeptide fragment or variant with a substrate molecule under conditions which allow the polypeptide fragment or variant to function, and detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between the polypeptide and substrate thereby identifying a fragment or variant of such sequences.

In another aspect, the invention provides a process for resolving an enantiomeric mixture of esters. The process comprises contacting the ester mixture with a hydrolase enzyme of the invention and recovering a mixture of esters enriched in one enantiomer and/or a mixture of alcohols enriched in the opposite enantiomer.

## BRIEF DESCRIPTION OF THE FIGURES

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

**Figure 1** is a block diagram of a computer system.

5        **Figure 2** is a flow diagram illustrating one embodiment of a process for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database.

**Figure 3** is a flow diagram illustrating one embodiment of a process in a computer for determining whether two sequences are homologous.

10       **Figure 4** is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence.

**Figure 5A** is an illustration of the full-length DNA sequence (SEQ ID NO:1) of BD021 of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.) for all sequences of the present invention.

5        **Figure 5B** is an illustration of the full-length amino acid sequence (SEQ ID NO:2) of BD021.

**Figure 6A** is an illustration of the full-length DNA sequence (SEQ ID NO:3) of BD213.

10       **Figure 6B** is an illustration of the full-length amino acid sequence (SEQ ID NO:4) of BD213.

**Figure 7A** is an illustration of the full-length DNA sequence (SEQ ID NO:5) of BD100.

**Figure 7B** is an illustration of the full-length amino acid sequence (SEQ ID NO:6) of BD100.

5        **Figure 8A** is an illustration of the full-length DNA sequence (SEQ ID NO:7) of BD100.



**Figure 8B** is an illustration of the full-length amino acid sequence (SEQ ID NO:6) of BD100.

**Figure 9A** is an illustration of the full-length DNA sequence (SEQ ID NO:9) of BD073.

5        **Figure 9B** is an illustration of the full-length amino acid sequence (SEQ ID NO:10) of BD073.

**Figure 10A** is an illustration of the full-length DNA sequence (SEQ ID NO:11) of BD094.

**Figure 10B** is an illustration of the full-length amino acid sequence (SEQ ID NO:12) of BD094.

**Figure 11A** is an illustration of the full-length DNA sequence (SEQ ID NO:13) of BD423.

**Figure 11B** is an illustration of the full-length amino acid sequence (SEQ ID NO:14) of BD423.

## DETAILED DESCRIPTION

The present invention relates to hydrolases and polynucleotides encoding them. As used herein, the term "hydrolase" refers to an enzyme capable of catalyzing the cleavage of a covalent bond with addition of a water molecule across the bond. The term "hydrolase" encompasses enzymes having esterase activity, *i.e.*, enzymes capable of hydrolyzing ester groups to organic acids and alcohols. The polynucleotides of the invention have been identified as encoding polypeptides having esterase activity.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references that are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference. In the case of inconsistencies, the present disclosure will prevail.

For purposes of the present invention, the following definitions will be used:

### Definitions

As used herein, the term "kinetic resolution" refers to a technique for separating enantiomers that relies upon the difference in the rates at which two enantiomers react when contacted with chiral reagent or catalyst. In "enzymatic kinetic resolution", the chiral catalyst is an enzyme.

The phrases "nucleic acid" or "nucleic acid sequence" as used herein refer to an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin.

A "coding sequence of" or a "nucleotide sequence encoding" a particular polypeptide or protein, is a nucleic acid sequence which is transcribed and translated into the polypeptide or protein when placed under the control of appropriate regulatory sequences.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as

well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

“Amino acid” or “amino acid sequence” as used herein refer to an oligopeptide, peptide, polypeptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring or synthetic molecules.

The term “polypeptide” as used herein, refers to amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of a phosphatidylinositol, cross-linking cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pergylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, and transfer-RNA mediated addition of amino acids to protein such as arginylation. (See Creighton, T.E., Proteins – Structure and Molecular Properties 2nd Ed., W.H. Freeman and Company, New York (1993); Posttranslational Covalent Modification of Proteins, B.C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983)).

As used herein, the term “isolated” refers to a material that has been removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector

and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. Individual nucleic acids obtained from a library have been conventionally purified to electrophoretic homogeneity. The sequences obtained from these clones could not be obtained directly either from the library or from total human DNA. The purified nucleic acids of the invention have been purified from the remainder of the genomic DNA in the organism by at least  $10^4$ - $10^6$  fold. However, the term "purified" also includes nucleic acids which have been purified from the remainder of the genomic DNA or from other sequences in a library or other environment by at least one order of magnitude, typically two or three orders, and more typically four or five orders of magnitude.

As used herein, the term "recombinant" means that the nucleic acid is adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. Additionally, to be "enriched" the nucleic acids will represent 5% or more of the number of nucleic acid inserts in a population of nucleic acid backbone molecules. Backbone molecules according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. Typically, the enriched nucleic acids represent 15% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. More typically, the enriched nucleic acids represent 50% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. In a one embodiment, the enriched nucleic acids represent 90% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules.

"Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide or protein. "Synthetic" polypeptides or protein are those prepared by chemical synthesis. Solid-phase chemical peptide synthesis methods can also be used to synthesize the polypeptide or fragments of the invention. Such method have been known in the art since the early 1960's (Merrifield, R. B., *J. Am. Chem. Soc.*, 85:2149-2154, 1963; see also Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed.,

Pierce Chemical Co., Rockford, Ill., pp. 11-12), and have recently been employed in commercially available laboratory peptide design and synthesis kits (Cambridge Research Biochemicals). Such commercially available laboratory kits have generally utilized the teachings of H. M. Geysen et al, *Proc. Natl. Acad. Sci.*, USA, 81:3998 (1984) and provide for synthesizing peptides upon the tips of a multitude of "rods" or "pins" all of which are connected to a single plate. When such a system is utilized, a plate of rods or pins is inverted and inserted into a second plate of corresponding wells or reservoirs, which contain solutions for attaching or anchoring an appropriate amino acid to the pin's or rod's tips. By repeating such a process step, i.e., inverting and inserting the rod's and pin's tips into appropriate solutions, amino acids are built into desired peptides. In addition, a number of available Fmoc peptide synthesis systems are available. For example, assembly of a polypeptide or fragment can be carried out on a solid support using an Applied Biosystems, Inc. Model 431A automated peptide synthesizer. Such equipment provides ready access to the peptides of the invention, either by direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

A promoter sequence is "operably linked to" a coding sequence when RNA polymerase which initiates transcription at the promoter will transcribe the coding sequence into mRNA.

"Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described herein are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 µg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 µl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 µg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by

the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion, gel electrophoresis may be performed to isolate the desired fragment.

“Oligonucleotide” refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

The phrase “substantially identical” in the context of two nucleic acids or polypeptides, refers to two or more sequences that have at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 99.9% nucleic acid or amino acid residue identity, respectively, when compared and aligned for maximum correspondence, as measured using one of the known sequence comparison algorithms or by visual inspection. Typically, the substantial identity exists over a region of at least about 100 residues, and most commonly the sequences are substantially identical over at least about 150-200 residues. In some embodiments, the sequences are substantially identical over the entire length of the coding regions.

Additionally a “substantially identical” amino acid sequence is a sequence that differs from a reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, provided that the polypeptide essentially retains its functional properties. Typically, such substitution, deletion or insertion is at a site that is not the active site of the molecule, but in some cases the substitution, deletion or insertion is at the active site. A conservative amino acid substitution, for example, substitutes one amino acid for another of the same class (e.g., substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid or glutamine for asparagine). One or more amino acids can be deleted, for example, from an hydrolase polypeptide, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for hydrolase biological activity can be removed. Modified

polypeptide sequences of the invention can be assayed for hydrolase biological activity by any number of methods, including contacting the modified polypeptide sequence with a hydrolase substrate and determining whether the modified polypeptide decreases the amount of specific substrate in the assay or increases the bioproducts of the enzymatic reaction of a functional hydrolase polypeptide with the substrate.

"Fragments" as used herein refers to polypeptides having the same, or substantially the same, amino acid sequence as a portion of a naturally occurring protein and retaining at least one functional activity of the protein to which it is related. An example of this is a "pro-form" molecule, such as a low activity proprotein that can be modified by cleavage to produce a mature enzyme with significantly higher activity.

"Hybridization" refers to the process by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

For example, hybridization under high stringency conditions could occur in about 50% formamide at about 37°C to 42°C. Hybridization could occur under reduced stringency conditions in about 35% to 25% formamide at about 30°C to 35°C. In particular, hybridization could occur under high stringency conditions at 42°C in 50% formamide, 5X SSPE, 0.3% SDS, and 200 n/ml sheared and denatured salmon sperm DNA. Hybridization could occur under reduced stringency conditions as described above, but in 35% formamide at a reduced temperature of 35°C. The temperature range corresponding to a particular level of stringency can be further narrowed by calculating the purine to pyrimidine ratio of the nucleic acid of interest and adjusting the temperature accordingly. Variations on the above ranges and conditions are well known in the art.

The term "about" is used herein to mean "approximately," or "roughly," or "around," or "in the region of." When the term "about" is used in conjunction with a numerical range, it

modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent. For example, "about 50%" refers to all values between 50% minus (0.2)(50) and 50% plus (0.2)(50), i.e., the range 40%-60%.

5       The term "variant" refers to polynucleotides or polypeptides of the invention modified at one or more base pairs, codons, introns, exons, or amino acid residues (respectively) yet still retaining the biological activity of an hydrolase of the invention. Variants can be produced by any number of means included methods such as, for example, error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential  
10 ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis (GSSM) and any combination thereof. In some embodiments, variants comprise non-natural nucleotides, e.g., inosine. In some embodiments, the modifications are repeated.

5       In one aspect, the present invention provides a non-stochastic method termed synthetic gene reassembly, that is somewhat related to stochastic shuffling, save that the nucleic acid building blocks are not shuffled or concatenated or chimerized randomly, but rather are assembled non-stochastically.

      The synthetic gene reassembly method does not depend on the presence of a high  
10 level of homology between polynucleotides to be shuffled. The invention can be used to non-stochastically generate libraries (or sets) of progeny molecules comprised of over  $10^{100}$  different chimeras. Conceivably, synthetic gene reassembly can even be used to generate libraries comprised of over  $10^{1000}$  different progeny chimeras.

      Thus, in one aspect, the invention provides a non-stochastic method of producing a set  
15 of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design, which method is comprised of the steps of generating by design a plurality of specific nucleic acid building blocks having serviceable mutually compatible ligatable ends, and assembling these nucleic acid building blocks, such that a designed overall assembly order is achieved.



The mutually compatible ligatable ends of the nucleic acid building blocks to be assembled are considered to be "serviceable" for this type of ordered assembly if they enable the building blocks to be coupled in predetermined orders. Thus, in one aspect, the overall assembly order in which the nucleic acid building blocks can be coupled is specified by the design of the ligatable ends and, if more than one assembly step is to be used, then the overall assembly order in which the nucleic acid building blocks can be coupled is also specified by the sequential order of the assembly step(s). In a one embodiment of the invention, the annealed building pieces are treated with an enzyme, such as a ligase (*e.g.*, T4 DNA ligase) to achieve covalent bonding of the building pieces.

In another embodiment, the design of nucleic acid building blocks is obtained upon analysis of the sequences of a set of progenitor nucleic acid templates that serve as a basis for producing a progeny set of finalized chimeric nucleic acid molecules. These progenitor nucleic acid templates thus serve as a source of sequence information that aids in the design of the nucleic acid building blocks that are to be mutagenized, *i.e.* chimerized or shuffled.

In one exemplification, the invention provides for the chimerization of a family of related genes and their encoded family of related products. In a particular exemplification, the encoded products are enzymes. The hydrolases of the present invention can be mutagenized in accordance with the methods described herein.

Thus according to one aspect of the invention, the sequences of a plurality of progenitor nucleic acid templates (*e.g.*, polynucleotides of Group A nucleic acid sequences) are aligned in order to select one or more demarcation points, which demarcation points can be located at an area of homology. The demarcation points can be used to delineate the boundaries of nucleic acid building blocks to be generated. Thus, the demarcation points identified and selected in the progenitor molecules serve as potential chimerization points in the assembly of the progeny molecules.

Typically a serviceable demarcation point is an area of homology (comprised of at least one homologous nucleotide base) shared by at least two progenitor templates, but the demarcation point can be an area of homology that is shared by at least half of the progenitor templates, at least two thirds of the progenitor templates, at least three fourths of the progenitor templates, and preferably at almost all of the progenitor templates. Even more

preferably still a serviceable demarcation point is an area of homology that is shared by all of the progenitor templates.

In a one embodiment, the gene reassembly process is performed exhaustively in order to generate an exhaustive library. In other words, all possible ordered combinations of the nucleic acid building blocks are represented in the set of finalized chimeric nucleic acid molecules. At the same time, the assembly order (*i.e.* the order of assembly of each building block in the 5' to 3' sequence of each finalized chimeric nucleic acid) in each combination is by design (or non-stochastic). Because of the non-stochastic nature of the method, the possibility of unwanted side products is greatly reduced.

In another embodiment, the method provides that the gene reassembly process is performed systematically, for example to generate a systematically compartmentalized library, with compartments that can be screened systematically, *e.g.*, one by one. In other words the invention provides that, through the selective and judicious use of specific nucleic acid building blocks, coupled with the selective and judicious use of sequentially stepped assembly reactions, an experimental design can be achieved where specific sets of progeny products are made in each of several reaction vessels. This allows a systematic examination and screening procedure to be performed. Thus, it allows a potentially very large number of progeny molecules to be examined systematically in smaller groups.

Because of its ability to perform chimerizations in a manner that is highly flexible yet exhaustive and systematic as well, particularly when there is a low level of homology among the progenitor molecules, the instant invention provides for the generation of a library (or set) comprised of a large number of progeny molecules. Because of the non-stochastic nature of the instant gene reassembly invention, the progeny molecules generated preferably comprise a library of finalized chimeric nucleic acid molecules having an overall assembly order that is chosen by design. In a particularly embodiment, such a generated library is comprised of greater than  $10^3$  to greater than  $10^{1000}$  different progeny molecular species.

In one aspect, a set of finalized chimeric nucleic acid molecules, produced as described is comprised of a polynucleotide encoding a polypeptide. According to one embodiment, this polynucleotide is a gene, which may be a man-made gene. According to another embodiment, this polynucleotide is a gene pathway, which may be a man-made gene

pathway. The invention provides that one or more man-made genes generated by the invention may be incorporated into a man-made gene pathway, such as pathway operable in a eukaryotic organism (including a plant).

5 In another exemplification, the synthetic nature of the step in which the building blocks are generated allows the design and introduction of nucleotides (*e.g.*, one or more nucleotides, which may be, for example, codons or introns or regulatory sequences) that can later be optionally removed in an *in vitro* process (*e.g.*, by mutagenesis) or in an *in vivo* process (*e.g.*, by utilizing the gene splicing ability of a host organism). It is appreciated that in many instances the introduction of these nucleotides may also be desirable for many other  
0 reasons in addition to the potential benefit of creating a serviceable demarcation point.

Thus, according to another embodiment, the invention provides that a nucleic acid building block can be used to introduce an intron. Accordingly, the invention provides that functional introns may be introduced into a man-made gene of the invention. The invention also provides that functional introns may be introduced into a man-made gene pathway of the  
5 invention. Accordingly, the invention provides for the generation of a chimeric polynucleotide that is a man-made gene containing one (or more) artificially introduced intron(s).

The invention also provides for the generation of a chimeric polynucleotide that is a man-made gene pathway containing one (or more) artificially introduced intron(s).  
0 Preferably, the artificially introduced intron(s) are functional in one or more host cells for gene splicing much in the way that naturally-occurring introns serve functionally in gene splicing. The invention provides a process of producing man-made intron-containing polynucleotides to be introduced into host organisms for recombination and/or splicing.

A man-made gene produced using the invention can also serve as a substrate for  
5 recombination with another nucleic acid. Likewise, a man-made gene pathway produced using the invention can also serve as a substrate for recombination with another nucleic acid. In a preferred instance, the recombination is facilitated by, or occurs at, areas of homology between the man-made, intron-containing gene and a nucleic acid, which serves as a recombination partner. In a particularly preferred instance, the recombination partner may  
3 also be a nucleic acid generated by the invention, including a man-made gene or a man-made

gene pathway. Recombination may be facilitated by or may occur at areas of homology that exist at the one (or more) artificially introduced intron(s) in the man-made gene.

The synthetic gene reassembly method of the invention utilizes a plurality of nucleic acid building blocks, each of which preferably has two ligatable ends. The two ligatable ends  
5 on each nucleic acid building block may be two blunt ends (*i.e.* each having an overhang of zero nucleotides), or preferably one blunt end and one overhang, or more preferably still two overhangs.

A useful overhang for this purpose may be a 3' overhang or a 5' overhang. Thus, a nucleic acid building block may have a 3' overhang or alternatively a 5' overhang or  
10 alternatively two 3' overhangs or alternatively two 5' overhangs. The overall order in which the nucleic acid building blocks are assembled to form a finalized chimeric nucleic acid molecule is determined by purposeful experimental design and is not random.

According to one preferred embodiment, a nucleic acid building block is generated by chemical synthesis of two single-stranded nucleic acids (also referred to as single-stranded  
5 oligos) and contacting them so as to allow them to anneal to form a double-stranded nucleic acid building block.

A double-stranded nucleic acid building block can be of variable size. The sizes of these building blocks can be small or large. Preferred sizes for building block range from 1 base pair (not including any overhangs) to 100,000 base pairs (not including any overhangs).  
10 Other preferred size ranges are also provided, which have lower limits of from 1 bp to 10,000 bp (including every integer value in between), and upper limits of from 2 bp to 100, 000 bp (including every integer value in between).

Many methods exist by which a double-stranded nucleic acid building block can be generated that is serviceable for the invention; and these are known in the art and can be  
5 readily performed by the skilled artisan.

According to one embodiment, a double-stranded nucleic acid building block is generated by first generating two single stranded nucleic acids and allowing them to anneal to form a double-stranded nucleic acid building block. The two strands of a double-stranded nucleic acid building block may be complementary at every nucleotide apart from any that

form an overhang; thus containing no mismatches, apart from any overhang(s). According to another embodiment, the two strands of a double-stranded nucleic acid building block are complementary at fewer than every nucleotide apart from any that form an overhang. Thus, according to this embodiment, a double-stranded nucleic acid building block can be used to  
5 introduce codon degeneracy. Preferably the codon degeneracy is introduced using the site-saturation mutagenesis described herein, using one or more N,N,G/T cassettes or alternatively using one or more N,N,N cassettes.

The *in vivo* recombination method of the invention can be performed blindly on a pool of unknown hybrids or alleles of a specific polynucleotide or sequence. However, it is  
1) not necessary to know the actual DNA or RNA sequence of the specific polynucleotide.

The approach of using recombination within a mixed population of genes can be useful for the generation of any useful proteins, for example, interleukin I, antibodies, tPA and growth hormone. This approach may be used to generate proteins having altered specificity or activity. The approach may also be useful for the generation of hybrid nucleic  
5 acid sequences, for example, promoter regions, introns, exons, enhancer sequences, 31 untranslated regions or 51 untranslated regions of genes. Thus this approach may be used to generate genes having increased rates of expression. This approach may also be useful in the study of repetitive DNA sequences. Finally, this approach may be useful to mutate ribozymes or aptamers.

1) In one aspect the invention described herein is directed to the use of repeated cycles of reductive reassortment, recombination and selection which allow for the directed molecular evolution of highly complex linear sequences, such as DNA, RNA or proteins thorough recombination.

*In vivo* shuffling of molecules is useful in providing variants and can be performed  
5 utilizing the natural property of cells to recombine multimers. While recombination *in vivo* has provided the major natural route to molecular diversity, genetic recombination remains a relatively complex process that involves 1) the recognition of homologies; 2) strand cleavage, strand invasion, and metabolic steps leading to the production of recombinant chiasma; and finally 3) the resolution of chiasma into discrete recombined molecules. The formation of the  
1) chiasma requires the recognition of homologous sequences.

In another embodiment, the invention includes a method for producing a hybrid polynucleotide from at least a first polynucleotide and a second polynucleotide. The invention can be used to produce a hybrid polynucleotide by introducing at least a first polynucleotide and a second polynucleotide which share at least one region of partial  
5 sequence homology into a suitable host cell. The regions of partial sequence homology promote processes which result in sequence reorganization producing a hybrid polynucleotide. The term "hybrid polynucleotide", as used herein, is any nucleotide sequence which results from the method of the present invention and contains sequence from at least two original polynucleotide sequences. Such hybrid polynucleotides can result from  
0 intermolecular recombination events which promote sequence integration between DNA molecules. In addition, such hybrid polynucleotides can result from intramolecular reductive reassortment processes which utilize repeated sequences to alter a nucleotide sequence within a DNA molecule.

The invention provides a means for generating hybrid polynucleotides which may  
5 encode biologically active hybrid polypeptides (*e.g.*, hybrid hydrolases). In one aspect, the original polynucleotides encode biologically active polypeptides. The method of the invention produces new hybrid polypeptides by utilizing cellular processes which integrate the sequence of the original polynucleotides such that the resulting hybrid polynucleotide encodes a polypeptide demonstrating activities derived from the original biologically active  
0 polypeptides. For example, the original polynucleotides may encode a particular enzyme from different microorganisms. An enzyme encoded by a first polynucleotide from one organism or variant may, for example, function effectively under a particular environmental condition, *e.g.* high salinity. An enzyme encoded by a second polynucleotide from a different organism or variant may function effectively under a different environmental condition, such  
5 as extremely high temperatures. A hybrid polynucleotide containing sequences from the first and second original polynucleotides may encode an enzyme which exhibits characteristics of both enzymes encoded by the original polynucleotides. Thus, the enzyme encoded by the hybrid polynucleotide may function effectively under environmental conditions shared by each of the enzymes encoded by the first and second polynucleotides, *e.g.*, high salinity and  
1) extreme temperatures.

Enzymes encoded by the polynucleotides of the invention include, but are not limited to, hydrolases, such as esterases. A hybrid polypeptide resulting from the method of the invention may exhibit specialized enzyme activity not displayed in the original enzymes. For example, following recombination and/or reductive reassortment of polynucleotides encoding hydrolase activities, the resulting hybrid polypeptide encoded by a hybrid polynucleotide can be screened for specialized hydrolase activities obtained from each of the original enzymes, i.e., the type of bond on which the hydrolase acts and the temperature at which the hydrolase functions. Thus, for example, the hydrolase may be screened to ascertain those chemical functionalities which distinguish the hybrid hydrolase from the original hydrolases, such as:

- ) (a) amide (peptide bonds), i.e., proteases; (b) ester bonds, i.e., esterases and lipases; (c) acetals, i.e., glycosidases and, for example, the temperature, pH or salt concentration at which the hybrid polypeptide functions.

Sources of the original polynucleotides may be isolated from individual organisms ("isolates"), collections of organisms that have been grown in defined media ("enrichment cultures"), or uncultivated organisms ("environmental samples"). The use of a culture-independent approach to derive polynucleotides encoding novel bioactivities from environmental samples is most preferable since it allows one to access untapped resources of biodiversity.

"Environmental libraries" are generated from environmental samples and represent the collective genomes of naturally occurring organisms archived in cloning vectors that can be propagated in suitable prokaryotic hosts. Because the cloned DNA is initially extracted directly from environmental samples, the libraries are not limited to the small fraction of prokaryotes that can be grown in pure culture. Additionally, a normalization of the environmental DNA present in these samples could allow more equal representation of the DNA from all of the species present in the original sample. This can dramatically increase the efficiency of finding interesting genes from minor constituents of the sample which may be under-represented by several orders of magnitude compared to the dominant species.

For example, gene libraries generated from one or more uncultivated microorganisms are screened for an activity of interest. Potential pathways encoding bioactive molecules of interest are first captured in prokaryotic cells in the form of gene expression libraries.

Polynucleotides encoding activities of interest are isolated from such libraries and introduced into a host cell. The host cell is grown under conditions which promote recombination and/or reductive reassortment creating potentially active biomolecules with novel or enhanced activities.

- 5       The microorganisms from which the polynucleotide may be prepared include prokaryotic microorganisms, such as *Eubacteria* and *Archaeobacteria*, and lower eukaryotic microorganisms such as fungi, some algae and protozoa. Polynucleotides may be isolated from environmental samples in which case the nucleic acid may be recovered without culturing of an organism or recovered from one or more cultured organisms. In one aspect,
- 0       such microorganisms may be extremophiles, such as hyperthermophiles, psychrophiles, psychrotrophs, halophiles, barophiles and acidophiles. Polynucleotides encoding enzymes isolated from extremophilic microorganisms are particularly preferred. Such enzymes may function at temperatures above 100°C in terrestrial hot springs and deep sea thermal vents, at temperatures below 0°C in arctic waters, in the saturated salt environment of the Dead Sea, at
- 5       pH values around 0 in coal deposits and geothermal sulfur-rich springs, or at pH values greater than 11 in sewage sludge. For example, several esterases and lipases cloned and expressed from extremophilic organisms show high activity throughout a wide range of temperatures and pHs.

- Polynucleotides selected and isolated as hereinabove described are introduced into a
- )       suitable host cell. A suitable host cell is any cell which is capable of promoting recombination and/or reductive reassortment. The selected polynucleotides are preferably already in a vector which includes appropriate control sequences. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or preferably, the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction
- 5       of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis *et al.*, 1986).

      As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or



Bowes melanoma; adenoviruses; and plant cells. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

With particular references to various mammalian cell culture systems that can be employed to express recombinant protein, examples of mammalian expression systems  
5 include the COS-7 lines of monkey kidney fibroblasts, described in "SV40-transformed simian cells support the replication of early SV40 mutants" (Gluzman, 1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites,  
10 polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Host cells containing the polynucleotides of interest can be cultured in conventional  
5 nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The clones which are identified as having the specified enzyme activity may then be sequenced to identify the polynucleotide sequence encoding an enzyme  
10 having the enhanced activity.

In another aspect, it is envisioned the method of the present invention can be used to generate novel polynucleotides encoding biochemical pathways from one or more operons or gene clusters or portions thereof. For example, bacteria and many eukaryotes have a coordinated mechanism for regulating genes whose products are involved in related  
5 processes. The genes are clustered, in structures referred to as "gene clusters," on a single chromosome and are transcribed together under the control of a single regulatory sequence, including a single promoter which initiates transcription of the entire cluster. Thus, a gene cluster is a group of adjacent genes that are either identical or related, usually as to their function. An example of a biochemical pathway encoded by gene clusters are polyketides.  
10 Polyketides are molecules which are an extremely rich source of bioactivities, including

antibiotics (such as tetracyclines and erythromycin), anti-cancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). Many polyketides (produced by polyketide synthases) are valuable as therapeutic agents. Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of an enormous variety of carbon chains differing in length and patterns of functionality and cyclization. Polyketide synthase genes fall into gene clusters and at least one type (designated type I) of polyketide synthases have large size genes and enzymes, complicating genetic manipulation and *in vitro* studies of these genes/proteins.

Gene cluster DNA can be isolated from different organisms and ligated into vectors, particularly vectors containing expression regulatory sequences which can control and regulate the production of a detectable protein or protein-related array activity from the ligated gene clusters. Use of vectors which have an exceptionally large capacity for exogenous DNA introduction are particularly appropriate for use with such gene clusters and are described by way of example herein to include the f-factor (or fertility factor) of *E. coli*. This f-factor of *E. coli* is a plasmid which affect high-frequency transfer of itself during conjugation and is ideal to achieve and stably propagate large DNA fragments, such as gene clusters from mixed microbial samples. A particularly preferred embodiment is to use cloning vectors, referred to as "fosmids" or bacterial artificial chromosome (BAC) vectors. These are derived from *E. coli* f-factor which is able to stably integrate large segments of genomic DNA. When integrated with DNA from a mixed uncultured environmental sample, this makes it possible to achieve large genomic fragments in the form of a stable "environmental DNA library." Another type of vector for use in the present invention is a cosmid vector. Cosmid vectors were originally designed to clone and propagate large segments of genomic DNA. Cloning into cosmid vectors is described in detail in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press (1989). Once ligated into an appropriate vector, two or more vectors containing different polyketide synthase gene clusters can be introduced into a suitable host cell. Regions of partial sequence homology shared by the gene clusters will promote processes which result in sequence reorganization resulting in a hybrid gene cluster. The novel hybrid gene cluster can then be screened for enhanced activities not found in the original gene clusters.

Therefore, in a one embodiment, the invention relates to a method for producing a biologically active hybrid polypeptide and screening such a polypeptide for enhanced activity by:

- 1) introducing at least a first polynucleotide in operable linkage and a second  
5 polynucleotide in operable linkage, said at least first polynucleotide and second polynucleotide sharing at least one region of partial sequence homology, into a suitable host cell;
- 2) growing the host cell under conditions which promote sequence reorganization resulting in a hybrid polynucleotide in operable linkage;
- 3) expressing a hybrid polypeptide encoded by the hybrid polynucleotide;
- 4) screening the hybrid polypeptide under conditions which promote  
identification of enhanced biological activity; and
- 5) isolating the a polynucleotide encoding the hybrid polypeptide.

Methods for screening for various enzyme activities are known to those of skill in the  
5 art and are discussed throughout the present specification. Such methods may be employed when isolating the polypeptides and polynucleotides of the invention.

As representative examples of expression vectors which may be used, there may be mentioned viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes, viral DNA (*e.g.*, vaccinia, adenovirus, retrovirus, adeno-  
associated virus, fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial  
chromosomes, yeast plasmids, yeast artificial chromosomes, and any other vectors specific  
for specific hosts of interest (such as bacillus, aspergillus and yeast). Thus, for example, the  
DNA may be included in any one of a variety of expression vectors for expressing a  
polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA  
5 sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, (lambda-ZAP vectors (Stratagene); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmid

or other vector may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct RNA synthesis. Particular named  
5 bacterial promoters include *lacI*, *lacZ*, *T3*, *T7*, *gpt*, *lambda P<sub>R</sub>*, *P<sub>L</sub>* and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The  
0 vector may also include appropriate sequences for amplifying expression. Promoter regions can be selected from any desired gene using chloramphenicol transferase (CAT) vectors or other vectors with selectable markers. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture,  
5 or such as tetracycline or ampicillin resistance in *E. coli*.

*In vivo* reassortment is focused on "inter-molecular" processes collectively referred to as "recombination" which in bacteria, is generally viewed as a "RecA-dependent" phenomenon. The invention can rely on recombination processes of a host cell to recombine and re-assort sequences, or the cells' ability to mediate reductive processes to decrease the  
0 complexity of quasi-repeated sequences in the cell by deletion. This process of "reductive reassortment" occurs by an "intra-molecular", RecA-independent process.

Therefore, in another aspect of the invention, novel polynucleotides can be generated by the process of reductive reassortment. The method involves the generation of constructs containing consecutive sequences (original encoding sequences), their insertion into an  
5 appropriate vector, and their subsequent introduction into an appropriate host cell. The reassortment of the individual molecular identities occurs by combinatorial processes between the consecutive sequences in the construct possessing regions of homology, or between quasi-repeated units. The reassortment process recombines and/or reduces the complexity and extent of the repeated sequences, and results in the production of novel  
0 molecular species. Various treatments may be applied to enhance the rate of reassortment.

These could include treatment with ultra-violet light, or DNA damaging chemicals, and/or the use of host cell lines displaying enhanced levels of "genetic instability". Thus the reassortment process may involve homologous recombination or the natural property of quasi-repeated sequences to direct their own evolution.

- 5 Repeated or "quasi-repeated" sequences play a role in genetic instability. In the present invention, "quasi-repeats" are repeats that are not restricted to their original unit structure. Quasi-repeated units can be presented as an array of sequences in a construct; consecutive units of similar sequences. Once ligated, the junctions between the consecutive sequences become essentially invisible and the quasi-repetitive nature of the resulting  
10 construct is now continuous at the molecular level. The deletion process the cell performs to reduce the complexity of the resulting construct operates between the quasi-repeated sequences. The quasi-repeated units provide a practically limitless repertoire of templates upon which slippage events can occur. The constructs containing the quasi-repeats thus effectively provide sufficient molecular elasticity that deletion (and potentially insertion)  
15 events can occur virtually anywhere within the quasi-repetitive units.

- When the quasi-repeated sequences are all ligated in the same orientation, for instance head to tail or vice versa, the cell cannot distinguish individual units. Consequently, the reductive process can occur throughout the sequences. In contrast, when for example, the units are presented head to head, rather than head to tail, the inversion delineates the  
20 endpoints of the adjacent unit so that deletion formation will favor the loss of discrete units. Thus, it is preferable with the present method that the sequences are in the same orientation. Random orientation of quasi-repeated sequences will result in the loss of reassortment efficiency, while consistent orientation of the sequences will offer the highest efficiency. However, while having fewer of the contiguous sequences in the same orientation decreases  
25 the efficiency, it may still provide sufficient elasticity for the effective recovery of novel molecules. Constructs can be made with the quasi-repeated sequences in the same orientation to allow higher efficiency.

Sequences can be assembled in a head to tail orientation using any of a variety of methods, including the following:

a) Primers that include a poly-A head and poly-T tail which when made single-stranded would provide orientation can be utilized. This is accomplished by having the first few bases of the primers made from RNA and hence easily removed RNaseH.

5 b) Primers that include unique restriction cleavage sites can be utilized. Multiple sites, a battery of unique sequences, and repeated synthesis and ligation steps would be required.

c) The inner few bases of the primer could be thiolated and an exonuclease used to produce properly tailed molecules.

0 The recovery of the re-assorted sequences relies on the identification of cloning vectors with a reduced repetitive index (RI). The re-assorted encoding sequences can then be recovered by amplification. The products are re-cloned and expressed. The recovery of cloning vectors with reduced RI can be affected by:

1) The use of vectors only stably maintained when the construct is reduced in complexity.

5 2) The physical recovery of shortened vectors by physical procedures. In this case, the cloning vector would be recovered using standard plasmid isolation procedures and size fractionated on either an agarose gel, or column with a low molecular weight cut off utilizing standard procedures.

0 3) The recovery of vectors containing interrupted genes which can be selected when insert size decreases.

4) The use of direct selection techniques with an expression vector and the appropriate selection.

5 Encoding sequences (for example, genes) from related organisms may demonstrate a high degree of homology and encode quite diverse protein products. These types of sequences are particularly useful in the present invention as quasi-repeats. However, while the examples illustrated below demonstrate the reassortment of nearly identical original encoding sequences (quasi-repeats), this process is not limited to such nearly identical repeats.

The following example demonstrates a method of the invention. Encoding nucleic acid sequences (quasi-repeats) derived from three (3) unique species are described. Each sequence encodes a protein with a distinct set of properties. Each of the sequences differs by a single or a few base pairs at a unique position in the sequence. The quasi-repeated sequences are separately or collectively amplified and ligated into random assemblies such that all possible permutations and combinations are available in the population of ligated molecules. The number of quasi-repeat units can be controlled by the assembly conditions. The average number of quasi-repeated units in a construct is defined as the repetitive index (RI).

Once formed, the constructs may, or may not be size fractionated on an agarose gel according to published protocols, inserted into a cloning vector, and transfected into an appropriate host cell. The cells are then propagated and "reductive reassortment" is effected. The rate of the reductive reassortment process may be stimulated by the introduction of DNA damage if desired. Whether the reduction in RI is mediated by deletion formation between repeated sequences by an "intra-molecular" mechanism, or mediated by recombination-like events through "inter-molecular" mechanisms is immaterial. The end result is a reassortment of the molecules into all possible combinations.

Optionally, the method comprises the additional step of screening the library members of the shuffled pool to identify individual shuffled library members having the ability to bind or otherwise interact, or catalyze a particular reaction (*e.g.*, such as catalytic domain of an enzyme) with a predetermined macromolecule, such as for example a proteinaceous receptor, an oligosaccharide, viron, or other predetermined compound or structure.

The polypeptides that are identified from such libraries can be used for therapeutic, diagnostic, research and related purposes (*e.g.*, catalysts, solutes for increasing osmolarity of an aqueous solution, and the like), and/or can be subjected to one or more additional cycles of shuffling and/or selection.

In another aspect, it is envisioned that prior to or during recombination or reassortment, polynucleotides generated by the method of the invention can be subjected to agents or processes which promote the introduction of mutations into the original polynucleotides. The introduction of such mutations would increase the diversity of resulting

hybrid polynucleotides and polypeptides encoded therefrom. The agents or processes which promote mutagenesis can include, but are not limited to: (+)-CC-1065, or a synthetic analog such as (+)-CC-1065-(N3-Adenine (*See* Sun and Hurley, (1992); an N-acetylated or deacetylated 4'-fluoro-4-aminobiphenyl adduct capable of inhibiting DNA synthesis (*See*, for example, van de Poll *et al.* (1992)); or a N-acetylated or deacetylated 4-aminobiphenyl adduct capable of inhibiting DNA synthesis (*See* also, van de Poll *et al.* (1992), pp. 751-758); trivalent chromium, a trivalent chromium salt, a polycyclic aromatic hydrocarbon (PAH) DNA adduct capable of inhibiting DNA replication, such as 7-bromomethyl-benz[a]anthracene ("BMA"), tris(2,3-dibromopropyl)phosphate ("Tris-BP"), 1,2-dibromo-3-chloropropane ("DBCP"), 2-bromoacrolein (2BA), benzo[a]pyrene-7,8-dihydrodiol-9-10-epoxide ("BPDE"), a platinum(II) halogen salt, N-hydroxy-2-amino-3-methylimidazo[4,5-f]quinoline ("N-hydroxy-IQ"), and N-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine ("N-hydroxy-PhIP"). Especially preferred means for slowing or halting PCR amplification consist of UV light (+)-CC-1065 and (+)-CC-1065-(N3-Adenine). Particularly encompassed means are DNA adducts or polynucleotides comprising the DNA adducts from the polynucleotides or polynucleotides pool, which can be released or removed by a process including heating the solution comprising the polynucleotides prior to further processing.

In another aspect the invention is directed to a method of producing recombinant proteins having biological activity by treating a sample comprising double-stranded template polynucleotides encoding a wild-type protein under conditions according to the invention which provide for the production of hybrid or re-assorted polynucleotides.

The invention also provides for the use of proprietary codon primers (containing a degenerate N,N,N sequence) to introduce point mutations into a polynucleotide, so as to generate a set of progeny polypeptides in which a full range of single amino acid substitutions is represented at each amino acid position (gene site saturated mutagenesis (GSSM)). The oligos used are comprised contiguously of a first homologous sequence, a degenerate N,N,N sequence, and preferably but not necessarily a second homologous sequence. The downstream progeny translational products from the use of such oligos include all possible amino acid changes at each amino acid site along the polypeptide, because the degeneracy of the N,N,N sequence includes codons for all 20 amino acids.



In one aspect, one such degenerate oligo (comprised of one degenerate N,N,N cassette) is used for subjecting each original codon in a parental polynucleotide template to a full range of codon substitutions. In another aspect, at least two degenerate N,N,N cassettes are used – either in the same oligo or not, for subjecting at least two original codons in a parental polynucleotide template to a full range of codon substitutions. Thus, more than one N,N,N sequence can be contained in one oligo to introduce amino acid mutations at more than one site. This plurality of N,N,N sequences can be directly contiguous, or separated by one or more additional nucleotide sequence(s). In another aspect, oligos serviceable for introducing additions and deletions can be used either alone or in combination with the codons containing an N,N,N sequence, to introduce any combination or permutation of amino acid additions, deletions, and/or substitutions.

In a particular exemplification, it is possible to simultaneously mutagenize two or more contiguous amino acid positions using an oligo that contains contiguous N,N,N triplets, *i.e.* a degenerate (N,N,N)<sub>n</sub> sequence.

In another aspect, the present invention provides for the use of degenerate cassettes having less degeneracy than the N,N,N sequence. For example, it may be desirable in some instances to use (*e.g.* in an oligo) a degenerate triplet sequence comprised of only one N, where said N can be in the first second or third position of the triplet. Any other bases including any combinations and permutations thereof can be used in the remaining two positions of the triplet. Alternatively, it may be desirable in some instances to use (*e.g.*, in an oligo) a degenerate N,N,N triplet sequence, N,N,G/T, or an N,N, G/C triplet sequence.

It is appreciated, however, that the use of a degenerate triplet (such as N,N,G/T or an N,N, G/C triplet sequence) as disclosed in the instant invention is advantageous for several reasons. In one aspect, this invention provides a means to systematically and fairly easily generate the substitution of the full range of possible amino acids (for a total of 20 amino acids) into each and every amino acid position in a polypeptide. Thus, for a 100 amino acid polypeptide, the invention provides a way to systematically and fairly easily generate 2000 distinct species (*i.e.*, 20 possible amino acids per position times 100 amino acid positions). It is appreciated that there is provided, through the use of an oligo containing a degenerate N,N,G/T or an N,N, G/C triplet sequence, 32 individual sequences that code for 20 possible

amino acids. Thus, in a reaction vessel in which a parental polynucleotide sequence is subjected to saturation mutagenesis using one such oligo, there are generated 32 distinct progeny polynucleotides encoding 20 distinct polypeptides. In contrast, the use of a non-degenerate oligo in site-directed mutagenesis leads to only one progeny polypeptide product  
5 per reaction vessel.

This invention also provides for the use of nondegenerate oligos, which can optionally be used in combination with degenerate primers disclosed. It is appreciated that in some situations, it is advantageous to use nondegenerate oligos to generate specific point mutations in a working polynucleotide. This provides a means to generate specific silent point  
) mutations, point mutations leading to corresponding amino acid changes, and point mutations that cause the generation of stop codons and the corresponding expression of polypeptide fragments.

Thus, in a preferred embodiment of this invention, each saturation mutagenesis reaction vessel contains polynucleotides encoding at least 20 progeny polypeptide molecules  
5 such that all 20 amino acids are represented at the one specific amino acid position corresponding to the codon position mutagenized in the parental polynucleotide. The 32-fold degenerate progeny polypeptides generated from each saturation mutagenesis reaction vessel can be subjected to clonal amplification (*e.g.*, cloned into a suitable *E. coli* host using an expression vector) and subjected to expression screening. When an individual progeny  
) polypeptide is identified by screening to display a favorable change in property (when compared to the parental polypeptide), it can be sequenced to identify the correspondingly favorable amino acid substitution contained therein.

It is appreciated that upon mutagenizing each and every amino acid position in a parental polypeptide using saturation mutagenesis as disclosed herein, favorable amino acid  
; changes may be identified at more than one amino acid position. One or more new progeny molecules can be generated that contain a combination of all or part of these favorable amino acid substitutions. For example, if two specific favorable amino acid changes are identified in each of 3 amino acid positions in a polypeptide, the permutations include 3 possibilities at each position (no change from the original amino acid, and each of two favorable changes)  
) and 3 positions. Thus, there are  $3 \times 3 \times 3$  or 27 total possibilities, including 7 that were

previously examined - 6 single point mutations (*i.e.*, 2 at each of three positions) and no change at any position.

In yet another aspect, site-saturation mutagenesis can be used together with shuffling, chimerization, recombination and other mutagenizing processes, along with screening. This invention provides for the use of any mutagenizing process(es), including saturation mutagenesis, in an iterative manner. In one exemplification, the iterative use of any mutagenizing process(es) is used in combination with screening.

Thus, in a non-limiting exemplification, this invention provides for the use of saturation mutagenesis in combination with additional mutagenization processes, such as process where two or more related polynucleotides are introduced into a suitable host cell such that a hybrid polynucleotide is generated by recombination and reductive reassortment.

In addition to performing mutagenesis along the entire sequence of a gene, the instant invention provides that mutagenesis can be used to replace each of any number of bases in a polynucleotide sequence, wherein the number of bases to be mutagenized is preferably every integer from 15 to 100,000. Thus, instead of mutagenizing every position along a molecule, one can subject every or a discrete number of bases (preferably a subset totaling from 15 to 100,000) to mutagenesis. Preferably, a separate nucleotide is used for mutagenizing each position or group of positions along a polynucleotide sequence. A group of 3 positions to be mutagenized may be a codon. The mutations are preferably introduced using a mutagenic primer, containing a heterologous cassette, also referred to as a mutagenic cassette. Preferred cassettes can have from 1 to 500 bases. Each nucleotide position in such heterologous cassettes be N, A, C, G, T, A/C, A/G, A/T, C/G, C/T, G/T, C/G/T, A/G/T, A/C/T, A/C/G, or E, where E is any base that is not A, C, G, or T (E can be referred to as a designer oligo).

In a general sense, saturation mutagenesis is comprised of mutagenizing a complete set of mutagenic cassettes (wherein each cassette is preferably about 1-500 bases in length) in defined polynucleotide sequence to be mutagenized (wherein the sequence to be mutagenized is preferably from about 15 to 100,000 bases in length). Thus, a group of mutations (ranging from 1 to 100 mutations) is introduced into each cassette to be mutagenized. A grouping of mutations to be introduced into one cassette can be different or the same from a second grouping of mutations to be introduced into a second cassette during the application of one

round of saturation mutagenesis. Such groupings are exemplified by deletions, additions, groupings of particular codons, and groupings of particular nucleotide cassettes.

Defined sequences to be mutagenized include a whole gene, pathway, cDNA, an entire open reading frame (ORF), and entire promoter, enhancer, repressor/transactivator, 5 origin of replication, intron, operator, or any polynucleotide functional group. Generally, a "defined sequences" for this purpose may be any polynucleotide that a 15 base-polynucleotide sequence, and polynucleotide sequences of lengths between 15 bases and 15,000 bases (this invention specifically names every integer in between). Considerations in choosing groupings of codons include types of amino acids encoded by a degenerate ) mutagenic cassette.

In a particularly preferred exemplification a grouping of mutations that can be introduced into a mutagenic cassette, this invention specifically provides for degenerate codon substitutions (using degenerate oligos) that code for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20 amino acids at each position, and a library of polypeptides 5 encoded thereby.

One aspect of the invention is an isolated nucleic acid comprising one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of a ) Group A nucleic acid sequence (or the sequences complementary thereto). The isolated, nucleic acids may comprise DNA, including cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. Alternatively, the isolated nucleic acids may comprise RNA.

5 As discussed in more detail below, the isolated nucleic acids of one of the Group A nucleic acid sequences, and sequences substantially identical thereto, may be used to prepare one of the polypeptides of a Group B amino acid sequence, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of Group B amino acid sequences, ) and sequences substantially identical thereto.

Accordingly, another aspect of the invention is an isolated nucleic acid which encodes one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of the Group B amino acid sequences. The coding sequences of these nucleic acids may be identical to one of the coding sequences of one of the nucleic acids of Group A nucleic acid sequences, or a fragment thereof or may be different coding sequences which encode one of the polypeptides of Group B amino acid sequences, sequences substantially identical thereto, and fragments having at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of one of the polypeptides of Group B amino acid sequences, as a result of the redundancy or degeneracy of the genetic code. The genetic code is well known to those of skill in the art and can be obtained, for example, on page 214 of B. Lewin, Genes VI, Oxford University Press, 1997, the disclosure of which is incorporated herein by reference.

The isolated nucleic acid which encodes one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, may include, but is not limited to: only the coding sequence of one of Group A nucleic acid sequences, and sequences substantially identical thereto, and additional coding sequences, such as leader sequences or proprotein sequences and non-coding sequences, such as introns or non-coding sequences 5' and/or 3' of the coding sequence. Thus, as used herein, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only the coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

Alternatively, the nucleic acid sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, may be mutagenized using conventional techniques, such as site directed mutagenesis, or other techniques familiar to those skilled in the art, to introduce silent changes into the polynucleotides of Group A nucleic acid sequences, and sequences substantially identical thereto. As used herein, "silent changes" include, for example, changes which do not alter the amino acid sequence encoded by the polynucleotide. Such changes may be desirable in order to increase the level of the polypeptide produced by host cells containing a vector encoding the polypeptide by introducing codons or codon pairs which occur frequently in the host organism.

The invention also relates to polynucleotides which have nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. Such nucleotide changes may be introduced using techniques such as site directed  
5 mutagenesis, random chemical mutagenesis, exonuclease III deletion, and other recombinant DNA techniques. Alternatively, such nucleotide changes may be naturally occurring allelic variants which are isolated by identifying nucleic acids which specifically hybridize to probes comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences  
0 substantially identical thereto (or the sequences complementary thereto) under conditions of high, moderate, or low stringency as provided herein.

The isolated nucleic acids of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500  
5 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or the sequences complementary thereto may also be used as probes to determine whether a biological sample, such as a soil sample, contains an organism having a nucleic acid sequence of the invention or an organism from which the nucleic acid was obtained. In such procedures, a biological sample potentially harboring the organism  
0 from which the nucleic acid was isolated is obtained and nucleic acids are obtained from the sample. The nucleic acids are contacted with the probe under conditions which permit the probe to specifically hybridize to any complementary sequences from which are present therein.

Where necessary, conditions which permit the probe to specifically hybridize to  
5 complementary sequences may be determined by placing the probe in contact with complementary sequences from samples known to contain the complementary sequence as well as control sequences which do not contain the complementary sequence. Hybridization conditions, such as the salt concentration of the hybridization buffer, the formamide concentration of the hybridization buffer, or the hybridization temperature, may be varied to  
0 identify conditions which allow the probe to hybridize specifically to complementary nucleic acids.

If the sample contains the organism from which the nucleic acid was isolated, specific hybridization of the probe is then detected. Hybridization may be detected by labeling the probe with a detectable agent such as a radioactive isotope, a fluorescent dye or an enzyme capable of catalyzing the formation of a detectable product.

- 5 Many methods for using the labeled probes to detect the presence of complementary nucleic acids in a sample are familiar to those skilled in the art. These include Southern Blots, Northern Blots, colony hybridization procedures, and dot blots. Protocols for each of these procedures are provided in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. (1997) and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual  
1) 2nd Ed., Cold Spring Harbor Laboratory Press (1989), the entire disclosures of which are incorporated herein by reference.

- Alternatively, more than one probe (at least one of which is capable of specifically hybridizing to any complementary sequences which are present in the nucleic acid sample), may be used in an amplification reaction to determine whether the sample contains an  
5 organism containing a nucleic acid sequence of the invention (*e.g.*, an organism from which the nucleic acid was isolated). Typically, the probes comprise oligonucleotides. In one embodiment, the amplification reaction may comprise a PCR reaction. PCR protocols are described in Ausubel and Sambrook, *supra*. Alternatively, the amplification may comprise a ligase chain reaction, 3SR, or strand displacement reaction. (See Barany, F., "The Ligase Chain  
1) Reaction in a PCR World", *PCR Methods and Applications* 1:5-16, 1991; E. Fahy *et al.*, "Self-sustained Sequence Replication (3SR): An Isothermal Transcription-based Amplification System Alternative to PCR", *PCR Methods and Applications* 1:25-33, 1991; and Walker G.T. *et al.*, "Strand Displacement Amplification-an Isothermal *in vitro* DNA Amplification Technique", *Nucleic Acid Research* 20:1691-1696, 1992, the disclosures of which are incorporated herein by  
5 reference in their entireties). In such procedures, the nucleic acids in the sample are contacted with the probes, the amplification reaction is performed, and any resulting amplification product is detected. The amplification product may be detected by performing gel electrophoresis on the reaction products and staining the gel with an intercalator such as ethidium bromide.  
Alternatively, one or more of the probes may be labeled with a radioactive isotope and the  
1) presence of a radioactive amplification product may be detected by autoradiography after gel electrophoresis.

Probes derived from sequences near the ends of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, may also be used in chromosome walking procedures to identify clones containing genomic sequences located adjacent to the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto. Such methods allow the isolation of genes which encode additional proteins from the host organism.

The isolated nucleic acids of Group A nucleic acid sequences, and sequences substantially identical thereto, the sequences complementary thereto, or a fragment comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or the sequences complementary thereto may be used as probes to identify and isolate related nucleic acids. In some embodiments, the related nucleic acids may be cDNAs or genomic DNAs from organisms other than the one from which the nucleic acid was isolated. For example, the other organisms may be related organisms. In such procedures, a nucleic acid sample is contacted with the probe under conditions which permit the probe to specifically hybridize to related sequences. Hybridization of the probe to nucleic acids from the related organism is then detected using any of the methods described above.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

Hybridization may be carried out under conditions of low stringency, moderate stringency or high stringency. As an example of nucleic acid hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 5.0 mM Na<sub>2</sub>EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/ml polyriboadenylic acid. Approximately 2 X 10<sup>7</sup> cpm (specific activity 4-9 X 10<sup>8</sup> cpm/ug) of <sup>32</sup>P end-labeled



oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na<sub>2</sub>EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T<sub>m</sub>-10°C for the oligonucleotide probe. The membrane is then  
 5 exposed to auto-radiographic film for detection of hybridization signals.

By varying the stringency of the hybridization conditions used to identify nucleic acids, such as cDNAs or genomic DNAs, which hybridize to the detectable probe, nucleic acids having different levels of homology to the probe can be identified and isolated. Stringency may be varied by conducting the hybridization at varying temperatures below the  
 ) melting temperatures of the probes. The melting temperature, T<sub>m</sub>, is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly complementary probe. Very stringent conditions are selected to be equal to or about 5°C lower than the T<sub>m</sub> for a particular probe. The melting temperature of the probe may be calculated using the following formulas:

5 For probes between 14 and 70 nucleotides in length the melting temperature (T<sub>m</sub>) is calculated using the formula:  $T_m = 81.5 + 16.6(\log [Na+]) + 0.41(\text{fraction G+C}) - (600/N)$  where N is the length of the probe.

If the hybridization is carried out in a solution containing formamide, the melting temperature may be calculated using the equation:  $T_m = 81.5 + 16.6(\log [Na+]) + 0.41(\text{fraction G+C}) - (0.63\% \text{ formamide}) - (600/N)$  where N is the length of the probe.  
 )

Prehybridization may be carried out in 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100µg denatured fragmented salmon sperm DNA or 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100µg denatured fragmented salmon sperm DNA, 50% formamide. The formulas for SSC and Denhardt's solutions are listed in Sambrook *et al.*, *supra*.

5 Hybridization is conducted by adding the detectable probe to the prehybridization solutions listed above. Where the probe comprises double stranded DNA, it is denatured before addition to the hybridization solution. The filter is contacted with the hybridization solution for a sufficient period of time to allow the probe to hybridize to cDNAs or genomic DNAs containing sequences complementary thereto or homologous thereto. For probes over

200 nucleotides in length, the hybridization may be carried out at 15-25°C below the  $T_m$ . For shorter probes, such as oligonucleotide probes, the hybridization may be conducted at 5-10°C below the  $T_m$ . Typically, for hybridizations in 6X SSC, the hybridization is conducted at approximately 68°C. Usually, for hybridizations in 50% formamide containing solutions,  
5 the hybridization is conducted at approximately 42°C.

All of the foregoing hybridizations would be considered to be under conditions of high stringency.

Following hybridization, the filter is washed to remove any non-specifically bound detectable probe. The stringency used to wash the filters can also be varied depending on the  
0 nature of the nucleic acids being hybridized, the length of the nucleic acids being hybridized, the degree of complementarity, the nucleotide sequence composition (*e.g.*, GC v. AT content), and the nucleic acid type (*e.g.*, RNA v. DNA). Examples of progressively higher stringency condition washes are as follows: 2X SSC, 0.1% SDS at room temperature for 15 minutes (low stringency); 0.1X SSC, 0.5% SDS at room temperature for 30 minutes to 1  
5 hour (moderate stringency); 0.1X SSC, 0.5% SDS for 15 to 30 minutes at between the hybridization temperature and 68°C (high stringency); and 0.15M NaCl for 15 minutes at 72°C (very high stringency). A final low stringency wash can be conducted in 0.1X SSC at room temperature. The examples above are merely illustrative of one set of conditions that can be used to wash filters. One of skill in the art would know that there are numerous  
0 recipes for different stringency washes. Some other examples are given below.

Nucleic acids which have hybridized to the probe are identified by autoradiography or other conventional techniques.

The above procedure may be modified to identify nucleic acids having decreasing levels of homology to the probe sequence. For example, to obtain nucleic acids of decreasing  
5 homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of 5°C from 68°C to 42°C in a hybridization buffer having a  $Na^+$  concentration of approximately 1M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate" conditions above 50°C and

“low” conditions below 50°C. A specific example of “moderate” hybridization conditions is when the above hybridization is conducted at 55°C. A specific example of “low stringency” hybridization conditions is when the above hybridization is conducted at 45°C.

- Alternatively, the hybridization may be carried out in buffers, such as 6X SSC,  
5 containing formamide at a temperature of 42°C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be “moderate” conditions above 25% formamide and “low” conditions below 25% formamide.
- ) A specific example of “moderate” hybridization conditions is when the above hybridization is conducted at 30% formamide. A specific example of “low stringency” hybridization conditions is when the above hybridization is conducted at 10% formamide.

- For example, the preceding methods may be used to isolate nucleic acids having a sequence with at least about 97%, at least 95%, at least 90%, at least 85%, at least 80%, at  
5 least 75%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% homology to a nucleic acid sequence selected from the group consisting of one of the sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, or fragments comprising at least about 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive bases thereof, and the sequences complementary thereto. Homology may be  
1) measured using the alignment algorithm. For example, the homologous polynucleotides may have a coding sequence which is a naturally occurring allelic variant of one of the coding sequences described herein. Such allelic variants may have a substitution, deletion or addition of one or more nucleotides when compared to the nucleic acids of Group A nucleic acid sequences or the sequences complementary thereto.

Additionally, the above procedures may be used to isolate nucleic acids which encode polypeptides having at least about 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% homology to a polypeptide having the sequence of one of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40,

50, 75, 100, or 150 consecutive amino acids thereof as determined using a sequence alignment algorithm (*e.g.*, such as the FASTA version 3.0t78 algorithm with the default parameters).

Another aspect of the invention is an isolated or purified polypeptide comprising the sequence of one of Group A nucleic acid sequences, and sequences substantially identical thereto, or fragments comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. As discussed above, such polypeptides may be obtained by inserting a nucleic acid encoding the polypeptide into a vector such that the coding sequence is operably linked to a sequence capable of driving the expression of the encoded polypeptide in a suitable host cell. For example, the expression vector may comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

Promoters suitable for expressing the polypeptide or fragment thereof in bacteria include the *E. coli lac* or *trp* promoters, the *lacI* promoter, the *lacZ* promoter, the *T3* promoter, the *T7* promoter, the *gpt* promoter, the *lambda P<sub>R</sub>* promoter, the *lambda P<sub>L</sub>* promoter, promoters from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), and the acid phosphatase promoter. Fungal promoters include the  $\alpha$  factor promoter. Eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, heat shock promoters, the early and late SV40 promoter, LTRs from retroviruses, and the mouse metallothionein-I promoter. Other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses may also be used.

Mammalian expression vectors may also comprise an origin of replication, any necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. In some embodiments, DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Vectors for expressing the polypeptide or fragment thereof in eukaryotic cells may also contain enhancers to increase expression levels. Enhancers are cis-acting elements of DNA, usually from about 10 to about 300 bp in length that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin

bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and the adenovirus enhancers.

In addition, the expression vectors typically contain one or more selectable marker genes to permit selection of host cells containing the vector. Such selectable markers include  
5 genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cell culture, genes conferring tetracycline or ampicillin resistance in *E. coli*, and the *S. cerevisiae TRP1* gene.

In some embodiments, the nucleic acid encoding one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising  
3 at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof is assembled in appropriate phase with a leader sequence capable of directing secretion of the translated polypeptide or fragment thereof. Optionally, the nucleic acid can encode a fusion polypeptide in which one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25,  
5 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof is fused to heterologous peptides or polypeptides, such as N-terminal identification peptides which impart desired characteristics, such as increased stability or simplified purification.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is ligated to the desired position in the vector  
3 following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, blunt ends in both the insert and the vector may be ligated. A variety of cloning techniques are disclosed in Ausubel *et al.* Current Protocols in Molecular Biology, John Wiley 503 Sons, Inc. 1997 and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press (1989), the entire disclosures of which are  
5 incorporated herein by reference. Such procedures and others are deemed to be within the scope of those skilled in the art.

The vector may be, for example, in the form of a plasmid, a viral particle, or a phage. Other vectors include chromosomal, nonchromosomal and synthetic DNA sequences, derivatives of SV40; bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors  
3 derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia,

adenovirus, fowl pox virus, and pseudorabies. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

- 5 Particular bacterial vectors which may be used include the commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), GEM1 (Promega Biotec, Madison, WI, USA) pQE70, pQE60, pQE-9 (Qiagen), pD10, psiX174 pBluescript II KS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, 0 pDR540, pRIT5 (Pharmacia), pKK232-8 and pCM7. Particular eukaryotic vectors include pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other vector may be used as long as it is replicable and viable in the host cell.

- The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells, eukaryotic cells, mammalian cells, insect cells, fungal cells, yeast cells, 5 plant cells, and metabolically rich host cells. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, fungal cells, such as yeast, insect cells such as *Drosophila S2* and *Spodoptera Sf9*, animal cells such as CHO, COS or Bowes melanoma, and adenoviruses. The 0 selection of an appropriate host is within the abilities of those skilled in the art.

- The vector may be introduced into the host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis, L., Dibner, M., Battey, 5 I., *Basic Methods in Molecular Biology*, (1986)).

- Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or 5 amplifying the genes of the invention. Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (*e.g.*, temperature shift or chemical induction) and the cells may be 0

cultured for an additional period to allow them to produce the desired polypeptide or fragment thereof.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells  
5 employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange  
1) chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

5 Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts (described by Gluzman, *Cell*, 23:175, 1981), and other cell lines capable of expressing proteins from a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines.

) The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Depending upon the host employed in a recombinant production procedure, the polypeptides produced by host cells containing the vector may be glycosylated or may be non-glycosylated. Polypeptides of the invention may or may not also include an initial methionine amino acid residue.

5 Alternatively, the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof can be synthetically produced by conventional peptide synthesizers. In other embodiments, fragments or portions of the polypeptides may be employed for producing the corresponding full-length polypeptide by

peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides.

Cell-free translation systems can also be employed to produce one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof using mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some embodiments, the DNA construct may be linearized prior to conducting an *in vitro* transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

The invention also relates to variants of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. The term "variant" includes derivatives or analogs of these polypeptides. In particular, the variants may differ in amino acid sequence from the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

The variants may be naturally occurring or created *in vitro*. In particular, such variants may be created using genetic engineering techniques such as site directed mutagenesis, random chemical mutagenesis, Exonuclease III deletion procedures, and standard cloning techniques. Alternatively, such variants, fragments, analogs, or derivatives may be created using chemical synthesis or modification procedures.

Other methods of making variants are also familiar to those skilled in the art. These include procedures in which nucleic acid sequences obtained from natural isolates are modified to generate nucleic acids which encode polypeptides having characteristics which enhance their value in industrial or laboratory applications. In such procedures, a large number of variant sequences having one or more nucleotide differences with respect to the sequence obtained from the natural isolate are generated and characterized. Typically, these nucleotide differences result in amino acid changes with respect to the polypeptides encoded by the nucleic acids from the natural isolates.



For example, variants may be created using error prone PCR. In error prone PCR, PCR is performed under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. Error prone PCR is described in Leung, D.W., *et al.*, *Technique*, 1:11-15, 1989) and  
5 Caldwell, R. C. & Joyce G.F., *PCR Methods Applic.*, 2:28-33, 1992, the disclosure of which is incorporated herein by reference in its entirety. Briefly, in such procedures, nucleic acids to be mutagenized are mixed with PCR primers, reaction buffer, MgCl<sub>2</sub>, MnCl<sub>2</sub>, Taq polymerase and an appropriate concentration of dNTPs for achieving a high rate of point mutation along the entire length of the PCR product. For example, the reaction may be  
0 performed using 20 fmoles of nucleic acid to be mutagenized, 30pmole of each PCR primer, a reaction buffer comprising 50 mM KCl, 10mM Tris HCl (pH 8.3) and 0.01% gelatin, 7 mM MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, 5 units of Taq polymerase, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, and 1 mM dTTP. PCR may be performed for 30 cycles of 94° C for 1 min, 45° C for 1 min, and 72° C for 1 min. However, it will be appreciated that these parameters may be  
5 varied as appropriate. The mutagenized nucleic acids are cloned into an appropriate vector and the activities of the polypeptides encoded by the mutagenized nucleic acids is evaluated.

Variants may also be created using oligonucleotide directed mutagenesis to generate site-specific mutations in any cloned DNA of interest. Oligonucleotide mutagenesis is described in Reidhaar-Olson, J.F. & Sauer, R.T., *et al.*, *Science*, 241:53-57, 1988, the  
10 disclosure of which is incorporated herein by reference in its entirety. Briefly, in such procedures a plurality of double stranded oligonucleotides bearing one or more mutations to be introduced into the cloned DNA are synthesized and inserted into the cloned DNA to be mutagenized. Clones containing the mutagenized DNA are recovered and the activities of the polypeptides they encode are assessed.

Another method for generating variants is assembly PCR. Assembly PCR involves the assembly of a PCR product from a mixture of small DNA fragments. A large number of different PCR reactions occur in parallel in the same vial, with the products of one reaction priming the products of another reaction. Assembly PCR is described in U.S. Patent No. 5,965,408, filed July 9, 1996, entitled, "Method of DNA Reassembly by Interrupting  
15 Synthesis", the disclosure of which is incorporated herein by reference in its entirety.

Still another method of generating variants is sexual PCR mutagenesis. In sexual PCR mutagenesis, forced homologous recombination occurs between DNA molecules of different but highly related DNA sequence *in vitro*, as a result of random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction. Sexual PCR mutagenesis is described in Stemmer, W.P., PNAS, USA, 91:10747-10751, 1994, the disclosure of which is incorporated herein by reference. Briefly, in such procedures a plurality of nucleic acids to be recombined are digested with DNase to generate fragments having an average size of 50-200 nucleotides. Fragments of the desired average size are purified and resuspended in a PCR mixture. PCR is conducted under conditions which facilitate recombination between the nucleic acid fragments. For example, PCR may be performed by resuspending the purified fragments at a concentration of 10-30ng/ $\mu$ l in a solution of 0.2 mM of each dNTP, 2.2 mM MgCl<sub>2</sub>, 50 mM KCL, 10 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100. 2.5 units of Taq polymerase per 100  $\mu$ l of reaction mixture is added and PCR is performed using the following regime: 94° C for 60 seconds, 94° C for 30 seconds, 50-55° C for 30 seconds, 72° C for 30 seconds (30-45 times) and 72° C for 5 minutes. However, it will be appreciated that these parameters may be varied as appropriate. In some embodiments, oligonucleotides may be included in the PCR reactions. In other embodiments, the Klenow fragment of DNA polymerase I may be used in a first set of PCR reactions and Taq polymerase may be used in a subsequent set of PCR reactions. Recombinant sequences are isolated and the activities of the polypeptides they encode are assessed.

Variants may also be created by *in vivo* mutagenesis. In some embodiments, random mutations in a sequence of interest are generated by propagating the sequence of interest in a bacterial strain, such as an E. coli strain, which carries mutations in one or more of the DNA repair pathways. Such "mutator" strains have a higher random mutation rate than that of a wild-type parent. Propagating the DNA in one of these strains will eventually generate random mutations within the DNA. Mutator strains suitable for use for *in vivo* mutagenesis are described in PCT Publication No. WO 91/16427, published October 31, 1991, entitled "Methods for Phenotype Creation from Multiple Gene Populations" the disclosure of which is incorporated herein by reference in its entirety.

Variants may also be generated using cassette mutagenesis. In cassette mutagenesis a small region of a double stranded DNA molecule is replaced with a synthetic oligonucleotide "cassette" that differs from the native sequence. The oligonucleotide often contains completely and/or partially randomized native sequence.

- 5 Recursive ensemble mutagenesis may also be used to generate variants. Recursive ensemble mutagenesis is an algorithm for protein engineering (protein mutagenesis) developed to produce diverse populations of phenotypically related mutants whose members differ in amino acid sequence. This method uses a feedback mechanism to control successive rounds of combinatorial cassette mutagenesis. Recursive ensemble mutagenesis is described  
0 in Arkin, A.P. and Youvan, D.C., *PNAS, USA*, 89:7811-7815, 1992, the disclosure of which is incorporated herein by reference in its entirety.

- In some embodiments, variants are created using exponential ensemble mutagenesis. Exponential ensemble mutagenesis is a process for generating combinatorial libraries with a high percentage of unique and functional mutants, wherein small groups of residues are  
5 randomized in parallel to identify, at each altered position, amino acids which lead to functional proteins. Exponential ensemble mutagenesis is described in Delegrave, S. and Youvan, D.C., *Biotechnology Research*, 11:1548-1552, 1993, the disclosure of which is incorporated herein by reference in its entirety. Random and site-directed mutagenesis are described in Arnold, F.H., *Current Opinions in Biotechnology*, 4:450-455, 1993, the  
0 disclosure of which is incorporated herein by reference in its entirety.

- In some embodiments, the variants are created using shuffling procedures wherein portions of a plurality of nucleic acids which encode distinct polypeptides are fused together to create chimeric nucleic acid sequences which encode chimeric polypeptides as described in U.S. Patent No. 5,965,408, filed July 9, 1996, entitled, "Method of DNA Reassembly by  
5 Interrupting Synthesis", and U.S. Patent No. 5,939,250, filed May 22, 1996, entitled, "Production of Enzymes Having Desired Activities by Mutagenesis," both of which are incorporated herein by reference.

- The variants of the polypeptides of Group B amino acid sequences may be variants in which one or more of the amino acid residues of the polypeptides of the Group B amino acid  
0 sequences are substituted with a conserved or non-conserved amino acid residue (preferably a

conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code.

Conservative substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative  
5 substitutions are the following replacements: replacements of an aliphatic amino acid such as Alanine, Valine, Leucine and Isoleucine with another aliphatic amino acid; replacement of a Serine with a Threonine or vice versa; replacement of an acidic residue such as Aspartic acid and Glutamic acid with another acidic residue; replacement of a residue bearing an amide group, such as Asparagine and Glutamine, with another residue bearing an amide group;  
0 exchange of a basic residue such as Lysine and Arginine with another basic residue; and replacement of an aromatic residue such as Phenylalanine, Tyrosine with another aromatic residue.

Other variants are those in which one or more of the amino acid residues of the polypeptides of the Group B amino acid sequences includes a substituent group.

5 Still other variants are those in which the polypeptide is associated with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol).

Additional variants are those in which additional amino acids are fused to the polypeptide, such as a leader sequence, a secretory sequence, a proprotein sequence or a  
0 sequence which facilitates purification, enrichment, or stabilization of the polypeptide.

In some embodiments, the fragments, derivatives and analogs retain the same biological function or activity as the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. In other embodiments, the fragment, derivative, or analog includes a proprotein, such that the fragment, derivative, or analog can be activated by  
5 cleavage of the proprotein portion to produce an active polypeptide.

Another aspect of the invention is polypeptides or fragments thereof which have at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or more than about 95% homology to one of the polypeptides of Group B amino

acid sequences, and sequences substantially identical thereto, or a fragment comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof.

Homology may be determined using any of the programs described above which aligns the polypeptides or fragments being compared and determines the extent of amino acid identity or similarity between them. It will be appreciated that amino acid "homology" includes conservative amino acid substitutions such as those described above.

The polypeptides or fragments having homology to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be obtained by isolating the nucleic acids encoding them using the techniques described above.

Alternatively, the homologous polypeptides or fragments may be obtained through biochemical enrichment or purification procedures. The sequence of potentially homologous polypeptides or fragments may be determined by proteolytic digestion, gel electrophoresis and/or microsequencing. The sequence of the prospective homologous polypeptide or fragment can be compared to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or a fragment comprising at least about 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof using any of the programs described above.

Another aspect of the invention is an assay for identifying fragments or variants of Group B amino acid sequences, and sequences substantially identical thereto, which retain the enzymatic function of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. For example the fragments or variants of said polypeptides, may be used to catalyze biochemical reactions, which indicate that the fragment or variant retains the enzymatic activity of the polypeptides in the Group B amino acid sequences.

The assay for determining if fragments of variants retain the enzymatic activity of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto includes the steps of: contacting the polypeptide fragment or variant with a substrate molecule under conditions which allow the polypeptide fragment or variant to function, and

detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between the polypeptide and substrate.

The polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or  
5 150 consecutive amino acids thereof, may be used to generate antibodies which bind specifically to the polypeptides or fragments. The resulting antibodies may be used in immunoaffinity chromatography procedures to isolate or purify the polypeptide or to determine whether the polypeptide is present in a biological sample. In such procedures, a protein preparation, such as an extract, or a biological sample is contacted with an antibody  
0 capable of specifically binding to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof.

In immunoaffinity procedures, the antibody is attached to a solid support, such as a bead or other column matrix. The protein preparation is placed in contact with the antibody  
5 under conditions in which the antibody specifically binds to one of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragment thereof. After a wash to remove non-specifically bound proteins, the specifically bound polypeptides are eluted.

“Specifically bind” means that an antibody binds to its target antigen (e.g., a  
0 polypeptide having hydrolase activity of the present invention) with greater affinity than it binds unrelated proteins. Preferably such affinity is at least 10-fold greater, more preferably at least 100-fold greater, and most preferably at least 1000-fold greater than the affinity of the antibody for unrelated proteins. Preferably, the antibody that specifically binds its target antigen forms an association with that antigen with an affinity of at least  $10^6 \text{ M}^{-1}$ , more  
5 preferably, at least  $10^7 \text{ M}^{-1}$ , even more preferably, at least  $10^8 \text{ M}^{-1}$ , even more preferably, at least  $10^9 \text{ M}^{-1}$ , and most preferably, at least  $10^{10} \text{ M}^{-1}$  either in water, under physiological conditions, or under conditions which approximate physiological conditions with respect to ionic strength, e.g., 140 mM NaCl, 5 mM  $\text{MgCl}_2$ .

The ability of proteins in a biological sample to specifically bind to the antibody may  
0 be determined using any of a variety of procedures familiar to those skilled in the art. For

example, binding may be determined by labeling the antibody with a detectable label such as a fluorescent agent, an enzymatic label, or a radioisotope. Alternatively, binding of the antibody to the sample may be detected using a secondary antibody having such a detectable label thereon. Particular assays include ELISA assays, sandwich assays, radioimmunoassays, and Western Blots.

Polyclonal antibodies generated against the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, for example, a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies which may bind to the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from cells expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, *Nature*, 256:495-497, 1975, the disclosure of which is incorporated herein by reference), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today* 4:72, 1983, the disclosure of which is incorporated herein by reference), and the EBV-hybridoma technique (Cole, *et al.*, 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, the disclosure of which is incorporated herein by reference).

Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778, the disclosure of which is incorporated herein by reference) can be adapted to produce single chain antibodies to the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof. Alternatively, transgenic mice may be used to express humanized antibodies to these polypeptides or fragments thereof.

Antibodies generated against the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be used in screening for similar polypeptides from other organisms and samples. In such techniques, polypeptides from the organism are contacted with the antibody and those polypeptides which specifically bind the antibody are detected. Any of the procedures described above may be used to detect antibody binding. One such screening assay is described in "Methods for Measuring Cellulase Activities", *Methods in Enzymology*, Vol 160, pp. 87-116, which is hereby incorporated by reference in its entirety.

As used herein the term "nucleic acid sequence as set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13" encompasses the nucleotide sequences of Group A nucleic acid sequences, and sequences substantially identical thereto, as well as sequences homologous to Group A nucleic acid sequences, and fragments thereof and sequences complementary to all of the preceding sequences. The fragments include portions of SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13, comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive nucleotides of Group A nucleic acid sequences, and sequences substantially identical thereto. Homologous sequences and fragments of Group A nucleic acid sequences, and sequences substantially identical thereto, refer to a sequence having at least 99.9%, 99.5%, 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% homology to these sequences. Homology may be determined using any of the computer programs and parameters described herein, including FASTA version 3.0t78 with the default parameters. Homologous sequences also include RNA sequences in which uridines replace the thymines in the nucleic acid sequences as set forth in the Group A nucleic acid sequences. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error. It will be appreciated that the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, can be represented in the traditional single character format (See the inside back cover of Stryer, Lubert. Biochemistry, 3rd Ed., W. H Freeman & Co., New York.) or in any other format which records the identity of the nucleotides in a sequence.

As used herein the term "a polypeptide sequence as set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, or 14" encompasses the polypeptide sequence of Group B amino acid sequences, and



sequences substantially identical thereto, which are encoded by a sequence as set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, or 14, polypeptide sequences homologous to the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto, or fragments of any of the preceding sequences. Homologous polypeptide sequences refer to a polypeptide  
5 sequence having at least 99.9%, 99.5%, 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% homology to one of the polypeptide sequences of the Group B amino acid sequences. Homology may be determined using any of the computer programs and parameters described herein, including FASTA version 3.0t78 with the default parameters or with any modified parameters. The homologous sequences may be obtained using any of the  
0 procedures described herein or may result from the correction of a sequencing error. The polypeptide fragments comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of the polypeptides of Group B amino acid sequences, and sequences substantially identical thereto. It will be appreciated that the polypeptide codes as set forth in Group B amino acid sequences, and sequences substantially identical, thereto, can be  
5 represented in the traditional single character format or three letter format (see the inside back cover of Stryer, Lubert. Biochemistry, 3rd Ed., W. H Freeman & Co., New York.) or in any other format which relates the identity of the polypeptides in a sequence.

It will be appreciated by those skilled in the art that a nucleic acid sequence as set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, or 13 and a polypeptide sequence as set forth in SEQ ID  
0 NOS:2, 4, 6, 8, 10, 12, or 14 can be stored, recorded, and manipulated on any medium which can be read and accessed by a computer. As used herein, the words "recorded" and "stored" refer to a process for storing information on a computer medium. A skilled artisan can readily adopt any of the presently known methods for recording information on a computer readable medium to generate manufactures comprising one or more of the nucleic acid sequences as set  
5 forth in Group A nucleic acid sequences, and sequences substantially identical thereto, one or more of the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, or 20 nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto.

0 Another aspect of the invention is a computer readable medium having recorded thereon one or more of the nucleic acid sequences as set forth in Group A nucleic acid

sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon one or more of the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto. Another aspect of the invention is a computer readable medium having recorded thereon at least  
5 2, 5, 10, 15, or 20 of the sequences as set forth above.

Computer readable media include magnetically readable media, optically readable media, electronically readable media and magnetic/optical media. For example, the computer readable media may be a hard disk, a floppy disk, a magnetic tape, CD-ROM, Digital Versatile Disk (DVD), Random Access Memory (RAM), or Read Only Memory (ROM) as  
0 well as other types of other media known to those skilled in the art.

Embodiments of the invention include systems (*e.g.*, internet based systems), particularly computer systems which store and manipulate the sequence information described herein. One example of a computer system 100 is illustrated in block diagram form in Figure 1. As used herein, "a computer system" refers to the hardware components,  
5 software components, and data storage components used to analyze a nucleotide sequence of a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in the Group B amino acid sequences. The computer system 100 typically includes a processor for processing, accessing and manipulating the sequence data. The processor 105 can be any well-known  
0 type of central processing unit, such as, for example, the Pentium III from Intel Corporation, or similar processor from Sun, Motorola, Compaq, AMD or International Business Machines.

Typically the computer system 100 is a general purpose system that comprises the processor 105 and one or more internal data storage components 110 for storing data, and one or more data retrieving devices for retrieving the data stored on the data storage components.  
5 A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

In one particular embodiment, the computer system 100 includes a processor 105 connected to a bus which is connected to a main memory 115 (preferably implemented as RAM) and one or more internal data storage devices 110, such as a hard drive and/or other  
0 computer readable media having data recorded thereon. In some embodiments, the computer

system 100 further includes one or more data retrieving device 118 for reading the data stored on the internal data storage devices 110.

5 The data retrieving device 118 may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, or a modem capable of connection to a remote data storage system (*e.g.*, via the internet) etc. In some embodiments, the internal data storage device 110 is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system 100 may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once  
0 inserted in the data retrieving device.

The computer system 100 includes a display 120 which is used to display output to a computer user. It should also be noted that the computer system 100 can be linked to other computer systems 125a-c in a network or wide area network to provide centralized access to the computer system 100.

5 Software for accessing and processing the nucleotide sequences of a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, (such as search tools, compare tools, and modeling tools etc.) may reside in main memory 115 during execution.

0 In some embodiments, the computer system 100 may further comprise a sequence comparison algorithm for comparing a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, stored on a computer readable medium to a reference nucleotide or polypeptide sequence(s) stored  
5 on a computer readable medium. A "sequence comparison algorithm" refers to one or more programs which are implemented (locally or remotely) on the computer system 100 to compare a nucleotide sequence with other nucleotide sequences and/or compounds stored within a data storage means. For example, the sequence comparison algorithm may compare the nucleotide sequences of a nucleic acid sequence as set forth in Group A nucleic acid  
0 sequences, and sequences substantially identical thereto, or a polypeptide sequence as set

forth in Group B amino acid sequences, and sequences substantially identical thereto, stored on a computer readable medium to reference sequences stored on a computer readable medium to identify homologies or structural motifs. Various sequence comparison programs identified elsewhere in this patent specification are particularly contemplated for use in this aspect of the invention. Protein and/or nucleic acid sequence homologies may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85(8):2444-2448, 1988; Altschul *et al.*, *J. Mol. Biol.* 215(3):403-410, 1990; Thompson *et al.*, *Nucleic Acids Res.* 22(2):4673-4680, 1994; Higgins *et al.*, *Methods Enzymol.* 266:383-402, 1996; Altschul *et al.*, *J. Mol. Biol.* 215(3):403-410, 1990; Altschul *et al.*, *Nature Genetics* 3:266-272, 1993).

Homology or identity is often measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various deletions, substitutions and other modifications. The terms "homology" and "identity" in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same when compared and aligned for maximum correspondence over a comparison window or designated region as measured using any number of sequence comparison algorithms or by manual alignment and visual inspection.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequence for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981, by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970, by the search for similarity method of person & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection. Other algorithms for determining homology or identity include, for example, in addition to a BLAST program (Basic Local Alignment Search Tool at the National Center for Biological Information), ALIGN, AMAS (Analysis of Multiply Aligned Sequences), AMPS (Protein Multiple Sequence Alignment), ASSET (Aligned Segment Statistical Evaluation Tool), BANDS, BESTSCOR, BIOSCAN (Biological Sequence Comparative Analysis Node), BLIMPS (BLOCKS IMPROVED Searcher), FASTA, Intervals & Points, BMB, CLUSTAL V, CLUSTAL W, CONSENSUS, LCONSENSUS, WCONSENSUS, Smith-Waterman algorithm, DARWIN, Las Vegas algorithm, FNAT (Forced Nucleotide Alignment Tool), Framealign, Framesearch, DYNAMIC, FILTER, FSAP (Fristensky Sequence Analysis Package), GAP (Global Alignment Program), GENAL, GIBBS, GenQuest, ISSC (Sensitive Sequence Comparison), LALIGN (Local Sequence Alignment), LCP (Local Content Program), MACAW (Multiple Alignment Construction & Analysis Workbench), MAP (Multiple Alignment Program), MBLKP, MBLKN, PIMA (Pattern-Induced Multi-sequence Alignment), SAGA (Sequence Alignment by Genetic Algorithm) and WHAT-IF. Such alignment programs can also be used to screen genome databases to identify polynucleotide sequences having substantially identical sequences. A number of genome databases are available, for example, a substantial portion of the human genome is available as part of the Human Genome Sequencing Project (J. Roach, [http://weber.u.Washington.edu/~roach/human\\_genome\\_progress2.html](http://weber.u.Washington.edu/~roach/human_genome_progress2.html)) (Gibbs, 1995). At

least twenty-one other genomes have already been sequenced, including, for example, *M. genitalium* (Fraser *et al.*, 1995), *M. jannaschii* (Bult *et al.*, 1996), *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (Blattner *et al.*, 1997), and yeast (*S. cerevisiae*) (Mewes *et al.*, 1997), and *D. melanogaster* (Adams *et al.*, 2000). Significant progress has also been made in  
5 sequencing the genomes of model organism, such as mouse, *C. elegans*, and *Arabidopsis sp.* Several databases containing genomic information annotated with some functional information are maintained by different organization, and are accessible via the internet, for example, <http://www.tigr.org/tdb>; <http://www.genetics.wisc.edu>; <http://genome-www.stanford.edu/~ball>; <http://hiv-web.lanl.gov>; <http://www.ncbi.nlm.nih.gov>;  
0 <http://www.ebi.ac.uk>; <http://Pasteur.fr/other/biology>; and <http://www.genome.wi.mit.edu>.

One example of a useful algorithm is BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402, 1997, and Altschul *et al.*, *J. Mol. Biol.* 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information  
5 (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for  
0 initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction  
5 are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences)  
0 uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as

defaults a wordlength of 3, and expectations (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N= -4, and a comparison of both strands.

5 The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873, 1993). One measure of similarity provided by BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a references sequence if the smallest sum probability in a comparison of the test  
0 nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

In one embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") In particular, five specific BLAST programs are used to perform the following task:

- 5 (1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;
- (2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;
- (3) BLASTX compares the six-frame conceptual translation products of a  
0 query nucleotide sequence (both strands) against a protein sequence database;
- (4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and
- (5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

5 The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (*i.e.*, aligned) by means of a scoring matrix, many of which are known in the art.

Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet *et al.*, *Science* 256:1443-1445, 1992; Henikoff and Henikoff, *Proteins* 17:49-61, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, *e.g.*, Schwartz and Dayhoff, eds., 1978, *Matrices for Detecting Distance Relationships: Atlas of Protein Sequence and Structure*, Washington: National Biomedical Research Foundation). BLAST programs are accessible through the U.S. National Library of Medicine, *e.g.*, at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

The parameters used with the above algorithms may be adapted depending on the sequence length and degree of homology studied. In some embodiments, the parameters may be the default parameters used by the algorithms in the absence of instructions from the user.

Figure 2 is a flow diagram illustrating one embodiment of a process 200 for comparing a new nucleotide or protein sequence with a database of sequences in order to determine the homology levels between the new sequence and the sequences in the database. The database of sequences can be a private database stored within the computer system 100, or a public database such as GENBANK that is available through the Internet.

The process 200 begins at a start state 201 and then moves to a state 202 wherein the new sequence to be compared is stored to a memory in a computer system 100. As discussed above, the memory could be any type of memory, including RAM or an internal storage device.

The process 200 then moves to a state 204 wherein a database of sequences is opened for analysis and comparison. The process 200 then moves to a state 206 wherein the first sequence stored in the database is read into a memory on the computer. A comparison is then performed at a state 210 to determine if the first sequence is the same as the second sequence. It is important to note that this step is not limited to performing an exact comparison between the new sequence and the first sequence in the database. Well-known methods are known to those of skill in the art for comparing two nucleotide or protein sequences, even if they are not identical. For example, gaps can be introduced into one sequence in order to raise the homology level between the two tested sequences. The parameters that control whether gaps or other features are introduced into a sequence during comparison are normally entered by the user of the computer system.



Once a comparison of the two sequences has been performed at the state 210, a determination is made at a decision state 210 whether the two sequences are the same. Of course, the term "same" is not limited to sequences that are absolutely identical. Sequences that are within the homology parameters entered by the user will be marked as "same" in the process 200.

If a determination is made that the two sequences are the same, the process 200 moves to a state 214 wherein the name of the sequence from the database is displayed to the user. This state notifies the user that the sequence with the displayed name fulfills the homology constraints that were entered. Once the name of the stored sequence is displayed to the user, the process 200 moves to a decision state 218 wherein a determination is made whether more sequences exist in the database. If no more sequences exist in the database, then the process 200 terminates at an end state 220. However, if more sequences do exist in the database, then the process 200 moves to a state 224 wherein a pointer is moved to the next sequence in the database so that it can be compared to the new sequence. In this manner, the new sequence is aligned and compared with every sequence in the database.

It should be noted that if a determination had been made at the decision state 212 that the sequences were not homologous, then the process 200 would move immediately to the decision state 218 in order to determine if any other sequences were available in the database for comparison.

Accordingly, one aspect of the invention is a computer system comprising a processor, a data storage device having stored thereon a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, a data storage device having retrievably stored thereon reference nucleotide sequences or polypeptide sequences to be compared to a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, and a sequence comparer for conducting the comparison. The sequence comparer may indicate a homology level between the sequences compared or identify structural motifs in the above described nucleic acid code of Group A nucleic acid sequences,

and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, or it may identify structural motifs in sequences which are compared to these nucleic acid codes and polypeptide codes. In some embodiments, the data storage device may have stored thereon the sequences of at least 2, 5, 10, 15, 20, 25, 30 or 40 or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

Another aspect of the invention is a method for determining the level of homology between a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, and a reference nucleotide sequence. The method including reading the nucleic acid code or the polypeptide code and the reference nucleotide or polypeptide sequence through the use of a computer program which determines homology levels and determining homology between the nucleic acid code or polypeptide code and the reference nucleotide or polypeptide sequence with the computer program. The computer program may be any of a number of computer programs for determining homology levels, including those specifically enumerated herein, (*e.g.*, BLAST2N with the default parameters or with any modified parameters). The method may be implemented using the computer systems described above. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30 or 40 or more of the above described nucleic acid sequences as set forth in the Group A nucleic acid sequences, or the polypeptide sequences as set forth in the Group B amino acid sequences through use of the computer program and determining homology between the nucleic acid codes or polypeptide codes and reference nucleotide sequences or polypeptide sequences.

Figure 3 is a flow diagram illustrating one embodiment of a process 250 in a computer for determining whether two sequences are homologous. The process 250 begins at a start state 252 and then moves to a state 254 wherein a first sequence to be compared is stored to a memory. The second sequence to be compared is then stored to a memory at a state 256. The process 250 then moves to a state 260 wherein the first character in the first sequence is read and then to a state 262 wherein the first character of the second sequence is

read. It should be understood that if the sequence is a nucleotide sequence, then the character would normally be either A, T, C, G or U. If the sequence is a protein sequence, then it is preferably in the single letter amino acid code so that the first and sequence sequences can be easily compared.

- 5           A determination is then made at a decision state 264 whether the two characters are the same. If they are the same, then the process 250 moves to a state 268 wherein the next characters in the first and second sequences are read. A determination is then made whether the next characters are the same. If they are, then the process 250 continues this loop until two characters are not the same. If a determination is made that the next two characters are
- 0           not the same, the process 250 moves to a decision state 274 to determine whether there are any more characters either sequence to read.

- If there are not any more characters to read, then the process 250 moves to a state 276 wherein the level of homology between the first and second sequences is displayed to the user. The level of homology is determined by calculating the proportion of characters
- 5           between the sequences that were the same out of the total number of sequences in the first sequence. Thus, if every character in a first 100 nucleotide sequence aligned with a every character in a second sequence, the homology level would be 100%.

- Alternatively, the computer program may be a computer program which compares the nucleotide sequences of a nucleic acid sequence as set forth in the invention, to one or more
- 0           reference nucleotide sequences in order to determine whether the nucleic acid code of Group A nucleic acid sequences, and sequences substantially identical thereto, differs from a reference nucleic acid sequence at one or more positions. Optionally such a program records the length and identity of inserted, deleted or substituted nucleotides with respect to the sequence of either the reference polynucleotide or a nucleic acid sequence as set forth in
- 5           Group A nucleic acid sequences, and sequences substantially identical thereto. In one embodiment, the computer program may be a program which determines whether a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, contains a single nucleotide polymorphism (SNP) with respect to a reference nucleotide sequence.

Accordingly, another aspect of the invention is a method for determining whether a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, differs at one or more nucleotides from a reference nucleotide sequence comprising the steps of reading the nucleic acid code and the reference nucleotide sequence through use of a computer program which identifies differences between nucleic acid sequences and identifying differences between the nucleic acid code and the reference nucleotide sequence with the computer program. In some embodiments, the computer program is a program which identifies single nucleotide polymorphisms. The method may be implemented by the computer systems described above and the method illustrated in Figure 3. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30, or 40 or more of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, and the reference nucleotide sequences through the use of the computer program and identifying differences between the nucleic acid codes and the reference nucleotide sequences with the computer program.

In other embodiments the computer based system may further comprise an identifier for identifying features within a nucleic acid sequence as set forth in the Group A nucleic acid sequences or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

An "identifier" refers to one or more programs which identifies certain features within a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. In one embodiment, the identifier may comprise a program which identifies an open reading frame in a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto.

Figure 4 is a flow diagram illustrating one embodiment of an identifier process 300 for detecting the presence of a feature in a sequence. The process 300 begins at a start state 302 and then moves to a state 304 wherein a first sequence that is to be checked for features is stored to a memory 115 in the computer system 100. The process 300 then moves to a state 306 wherein a database of sequence features is opened. Such a database would include a list of each feature's attributes along with the name of the feature. For example, a feature

name could be "Initiation Codon" and the attribute would be "ATG". Another example would be the feature name "TAATAA Box" and the feature attribute would be "TAATAA". An example of such a database is produced by the University of Wisconsin Genetics Computer Group ([www.gcg.com](http://www.gcg.com)). Alternatively, the features may be structural polypeptide motifs such as alpha helices, beta sheets, or functional polypeptide motifs such as enzymatic active sites, helix-turn-helix motifs or other motifs known to those skilled in the art.

Once the database of features is opened at the state 306, the process 300 moves to a state 308 wherein the first feature is read from the database. A comparison of the attribute of the first feature with the first sequence is then made at a state 310. A determination is then made at a decision state 316 whether the attribute of the feature was found in the first sequence. If the attribute was found, then the process 300 moves to a state 318 wherein the name of the found feature is displayed to the user.

The process 300 then moves to a decision state 320 wherein a determination is made whether more features exist in the database. If no more features do exist, then the process 300 terminates at an end state 324. However, if more features do exist in the database, then the process 300 reads the next sequence feature at a state 326 and loops back to the state 310 wherein the attribute of the next feature is compared against the first sequence.

It should be noted, that if the feature attribute is not found in the first sequence at the decision state 316, the process 300 moves directly to the decision state 320 in order to determine if any more features exist in the database.

Accordingly, another aspect of the invention is a method of identifying a feature within a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, comprising reading the nucleic acid code(s) or polypeptide code(s) through the use of a computer program which identifies features therein and identifying features within the nucleic acid code(s) with the computer program. In one embodiment, computer program comprises a computer program which identifies open reading frames. The method may be performed by reading a single sequence or at least 2, 5, 10, 15, 20, 25, 30, or 40 of the nucleic acid sequences as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or the polypeptide

sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto, through the use of the computer program and identifying features within the nucleic acid codes or polypeptide codes with the computer program.

A nucleic acid sequence as set forth in Group A nucleic acid sequences, and  
 5 sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto, may be stored and manipulated in a variety of data processor programs in a variety of formats. For example, a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid  
 0 sequences, and sequences substantially identical thereto, may be stored as text in a word processing file, such as MicrosoftWORD or WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2, SYBASE, or ORACLE. In addition, many computer programs and databases may be used as sequence comparison algorithms, identifiers, or sources of reference nucleotide sequences or polypeptide sequences to  
 5 be compared to a nucleic acid sequence as set forth in Group A nucleic acid sequences, and sequences substantially identical thereto, or a polypeptide sequence as set forth in Group B amino acid sequences, and sequences substantially identical thereto. The following list is intended not to limit the invention but to provide guidance to programs and databases which are useful with the nucleic acid sequences as set forth in Group A nucleic acid sequences, and  
 0 sequences substantially identical thereto, or the polypeptide sequences as set forth in Group B amino acid sequences, and sequences substantially identical thereto.

The programs and databases which may be used include, but are not limited to:  
 MacPattern (EMBL), DiscoveryBase (Molecular Applications Group), GeneMine (Molecular Applications Group), Look (Molecular Applications Group), MacLook (Molecular  
 5 Applications Group), BLAST and BLAST2 (NCBI), BLASTN and BLASTX (Altschul et al, *J. Mol. Biol.* 215: 403, 1990), FASTA (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85: 2444, 1988), FASTDB (Brutlag et al. *Comp. App. Biosci.* 6:237-245, 1990), Catalyst (Molecular Simulations Inc.), Catalyst/SHAPE (Molecular Simulations Inc.),  
 Cerius<sup>2</sup>.DBAccess (Molecular Simulations Inc.), HypoGen (Molecular Simulations Inc.),  
 0 Insight II, (Molecular Simulations Inc.), Discover (Molecular Simulations Inc.), CHARMm (Molecular Simulations Inc.), Felix (Molecular Simulations Inc.), DelPhi, (Molecular

Simulations Inc.), QuanteMM, (Molecular Simulations Inc.), Homology (Molecular Simulations Inc.), Modeler (Molecular Simulations Inc.), ISIS (Molecular Simulations Inc.), Quanta/Protein Design (Molecular Simulations Inc.), WebLab (Molecular Simulations Inc.), WebLab Diversity Explorer (Molecular Simulations Inc.), Gene Explorer (Molecular Simulations Inc.), SeqFold (Molecular Simulations Inc.), the MDL Available Chemicals Directory database, the MDL Drug Data Report data base, the Comprehensive Medicinal Chemistry database, Derwent's World Drug Index database, the BioByteMasterFile database, the Genbank database, and the Genseqn database. Many other programs and data bases would be apparent to one of skill in the art given the present disclosure.

0 Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

5 Enzymes are highly selective catalysts. Their hallmark is the ability to catalyze reactions with exquisite stereo-, regio-, and chemo- selectivities that are unparalleled in conventional synthetic chemistry. Moreover, enzymes are remarkably versatile. They can be tailored to function in organic solvents, operate at extreme pHs (for example, high pHs and low pHs) extreme temperatures (for example, high temperatures and low temperatures),  
0 extreme salinity levels (for example, high salinity and low salinity), and catalyze reactions with compounds that are structurally unrelated to their natural, physiological substrates.

Enzymes are reactive toward a wide range of natural and unnatural substrates, thus enabling the modification of virtually any organic lead compound. Moreover, unlike traditional chemical catalysts, enzymes are highly enantio- and regio-selective. The high  
5 degree of functional group specificity exhibited by enzymes enables one to keep track of each reaction in a synthetic sequence leading to a new active compound. Enzymes are also capable of catalyzing many diverse reactions unrelated to their physiological function in nature. For example, peroxidases catalyze the oxidation of phenols by hydrogen peroxide. Peroxidases can also catalyze hydroxylation reactions that are not related to the native function of the  
0 enzyme. Other examples are proteases which catalyze the breakdown of polypeptides. In

organic solution some proteases can also acylate sugars, a function unrelated to the native function of these enzymes.

The present invention exploits the unique catalytic properties of enzymes. Whereas the use of biocatalysts (i.e., purified or crude enzymes, non-living or living cells) in chemical transformations normally requires the identification of a particular biocatalyst that reacts with a specific starting compound, the present invention uses selected biocatalysts and reaction conditions that are specific for functional groups that are present in many starting compounds, such as small molecules. Each biocatalyst is specific for one functional group, or several related functional groups, and can react with many starting compounds containing this functional group.

The biocatalytic reactions produce a population of derivatives from a single starting compound. These derivatives can be subjected to another round of biocatalytic reactions to produce a second population of derivative compounds. Thousands of variations of the original small molecule or compound can be produced with each iteration of biocatalytic derivatization.

Enzymes react at specific sites of a starting compound without affecting the rest of the molecule, a process which is very difficult to achieve using traditional chemical methods. This high degree of biocatalytic specificity provides the means to identify a single active compound within the library. The library is characterized by the series of biocatalytic reactions used to produce it, a so called "biosynthetic history." Screening the library for biological activities and tracing the biosynthetic history identifies the specific reaction sequence producing the active compound. The reaction sequence is repeated and the structure of the synthesized compound determined. This mode of identification, unlike other synthesis and screening approaches, does not require immobilization technologies, and compounds can be synthesized and tested free in solution using virtually any type of screening assay. It is important to note, that the high degree of specificity of enzyme reactions on functional groups allows for the "tracking" of specific enzymatic reactions that make up the biocatalytically produced library.

Many of the procedural steps are performed using robotic automation enabling the execution of many thousands of biocatalytic reactions and screening assays per day as well as



ensuring a high level of accuracy and reproducibility. As a result, a library of derivative compounds can be produced in a matter of weeks which would take years to produce using current chemical methods. (For further teachings on modification of molecules, including small molecules, see PCT/US94/09174, herein incorporated by reference in its entirety).

5           In a particular embodiment, the invention provides a method for modifying small molecules, comprising contacting a polypeptide encoded by a polynucleotide described herein or enzymatically active fragments thereof with a small molecule to produce a modified small molecule. A library of modified small molecules is tested to determine if a modified small molecule is present within the library which exhibits a desired activity. A  
0   specific biocatalytic reaction which produces the modified small molecule of desired activity is identified by systematically eliminating each of the biocatalytic reactions used to produce a portion of the library, and then testing the small molecules produced in the portion of the library for the presence or absence of the modified small molecule with the desired activity. The specific biocatalytic reactions which produce the modified small molecule of desired  
5   activity is optionally repeated. The biocatalytic reactions are conducted with a group of biocatalysts that react with distinct structural moieties found within the structure of a small molecule, each biocatalyst is specific for one structural moiety or a group of related structural moieties; and each biocatalyst reacts with many different small molecules which contain the distinct structural moiety.

0           The polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids thereof may be used in a variety of applications. For example, the polypeptides or fragments thereof may be used to catalyze biochemical reactions. In accordance with one aspect of the invention, there is provided a process for utilizing the  
5   polypeptides of Group B amino acid sequences, and sequences substantially identical thereto or polynucleotides encoding such polypeptides for hydrolyzing glycosidic linkages. In such procedures, a substance containing a glycosidic linkage (*e.g.*, a starch) is contacted with one of the polypeptides of Group B amino acid sequences, or sequences substantially identical thereto under conditions which facilitate the hydrolysis of the glycosidic linkage.

The polypeptides described herein can also be used for the enzymatic kinetic resolution of enantiomeric mixtures. In another aspect, therefore, the invention provides a process for resolving a substrate enantiomeric mixture by contacting the substrate with a hydrolase enzyme of the invention and recovering the substrate and/or the product in enantiomerically enriched form.

In one embodiment, the substrate is an enantiomeric mixture of esters, and the hydrolase enzyme catalyzes the hydrolysis of the ester to an alcohol and an acid. In certain preferred embodiments, the ester is an alkanoate ester, preferably a C<sub>1</sub>-C<sub>6</sub> alkanoate ester, of a secondary alcohol. Nonlimiting examples of alkanoate esters that can be resolved according to this aspect of the invention include acetates and butyrates. In certain other preferred embodiments, the ester is an alkyl ester, preferably a C<sub>1</sub>-C<sub>20</sub>, C<sub>1</sub>-C<sub>12</sub>, C<sub>1</sub>-C<sub>6</sub>, or C<sub>1</sub>-C<sub>4</sub> alkyl ester, of a chiral organic acid. Nonlimiting examples of alkyl esters that can be resolved according to this aspect of the invention include methyl, ethyl, propyl, and butyl esters.

In another embodiment, the substrate is an enantiomeric mixture of alcohols, preferably secondary alcohols, and the hydrolase enzyme catalyzes the reaction of the alcohol with an acyl donor to form an ester. Vinyl acetate is an example of an acyl donor suitable for use in the esterification reaction, but others may also be used.

In some embodiments, the carbon atom to which the alcohol or ester is attached is the chiral center to be resolved. In other embodiments, the carbon atom to which the alcohol or ester is attached is achiral, and the chiral center to be resolved is at a different position in the molecule.

For example, the enzymes of the invention can be used to resolve an alcohol selected from the group consisting of 2-hydroxy-3,3-dimethyl- $\gamma$ -butyrolactone, 3-butyne-2-ol, 1-methoxy-2-propanol, and 3-hydroxytetrahydrofuran, or an ester thereof. These compounds are important chiral building blocks for organic synthesis. For instance, Nakamura *et al.*, *Tetrahedron: Asymmetry* 9:4429 (1998), discloses that 3-butyne-2-ol can be used for the synthesis of fenleuton, the sex pheromone of a male mountain pine beetle, and Cotteril *et al.*, *J. Chem. Soc. Perkin Trans. 1* 3269 (1994), reports that the same compound can be used for the synthesis of (*S*)-(-)-austrocorticin, the major pigment in the fruit bodies of an Australian

toadstool. Kataoka *et al.*, *Appl. Microbiol. Biotechnol.* 44:333 (1995), discloses that pantolactone (2-hydroxy-3,3-dimethyl- $\gamma$ -butyrolactone) is the precursor of D-pantothenic acid (vitamin B<sub>5</sub>). Ghosh *et al.*, *J. Med. Chem.* 39:3278 (1996), and Talipov *et al.*, *Russ. J. Org. Chem.* 29:1205 (1993). In each case, the enantiomeric purity of the product is important for biological activity, and methods for obtaining the chiral building blocks in enantiomerically enriched form are therefore needed.

The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

## EXAMPLES

### 0 Example 1: Isolation of Hydrolases

#### BD100

In accordance with the invention, the BD100 hydrolase was isolated from *Metallosphaera prunae*, a member of the Crenarchaeota kingdom having aerobic metabolism. *Metallosphaera prunae* was grown in culture conditions of pH 2 at 65°C.

5 To isolate BD100, a colimetric plate assay was used (using X-butyrate as a substrate). The assay was performed at 39°C, pH 7.5. The host organism was XL1-Blue MRF' (VPN200158). To isolate the BD100 clone, a gt11 library was screened. BD100 was PCR amplified from gt11, and TOPO-TA was cloned into pCR2.1, thereby forming the vector pCR2.1-TOPO (host TG1).

#### 3 BD073

In accordance with the invention, the BD073 hydrolase was isolated from *Alicyclobacillus acidocaldarius*, a bacillus bacterium which grows at 75°C, pH 6.0 and was isolated from Yellowstone, Wyoming, USA. *Alicyclobacillus acidocaldarius*, an aerobic heterotroph, was grown in culture conditions of pH 3.5 at 62°C.

5 To isolate BD073, a colimetric plate assay was used (using X-butyrate as a substrate). The assay was performed at 39°C, pH 7.5. The host organism was XL1-Blue MRF' (VPN200158). The isolated BD073 clone was subcloned into the lambda ZAPExpress vector.

**Example 2: Bacterial Expression and Purification of Hydrolases**

DNA encoding the enzymes of the present invention, SEQ ID NOS:1, 3, 5, 7, 9, 11, and 13, were initially amplified from a pBluescript vector containing the DNA by the PCR technique using a pQE vector. The pQE vector encodes antibiotic resistance (Amp<sup>r</sup>), a  
5 bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6His tag and restriction enzyme sites.

The pQE vector was digested with the restriction enzymes. The amplified sequences were ligated into the respective pQE vector and inserted in frame with the sequence encoding for the RBS. The ligation mixture was then used to transform the *E. coli* strain M15/pREP4  
0 (Qiagen, Inc.) by electroporation. M15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants were identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid  
5 culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells  
0 were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation.

The primer sequences set out above may also be employed to isolate the target gene from the deposited material by hybridization techniques described above.

**Example 3: Isolation of a Selected Clone from the Deposited Genomic Clones**

The two oligonucleotide primers corresponding to the gene of interest are used to  
5 amplify the gene from the deposited material. A polymerase chain reaction is carried out in 25 µl of reaction mixture with 0.1 µg of the DNA of the gene of interest. The reaction mixture is 1.5-5 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 20 µM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 1.25 Unit of Taq polymerase. Thirty cycles of PCR (denaturation at 94° C. for 1 min; annealing at 55° C. for 1 min; elongation at 72° C. for 1 min) are performed  
0 with the Perkin-Elmer Cetus 9600 thermal cycler. The amplified product is analyzed by

agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the gene of interest by subcloning and sequencing the DNA product.

#### **Example 4: Production of the Expression Gene Bank**

5 Colonies containing pBluescript plasmids with random inserts from the organisms M11TL, *Thermococcus* GU5L5, and *Teredinibacter* were obtained according to the method of Hay and Short, *Strategies*, 5:16, 1992.

#### **Example 5: Screening for Lipase/Esterase Activity**

0 The resulting colonies were picked with sterile toothpicks and used to singly inoculate each of the wells of 96-well microtiter plates. The wells contained 250  $\mu$ L of LB media with 100  $\mu$ g/mL ampicillin, 80  $\mu$ g/mL methicillin, and 10% v/v glycerol (LB Amp/Meth, glycerol). The cells were grown overnight at 37° C. without shaking. This constituted generation of the "Source GeneBank." Each well of the Source GeneBank thus contained a stock culture of *E. coli* cells, each of which contained a pBluescript with a unique DNA  
5 insert.

The plates of the Source GeneBank were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200  $\mu$ L of LB Amp/Meth, glycerol. This step was performed using the High Density Replicating Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each  
0 well of the Condensed Plate thus contained 10 to 12 different pBluescript clones from each of the source library plates. The Condensed Plate was grown for 16 hours at 37° C. and then used to inoculate two white 96-well Polyfiltronics microtiter daughter plates containing in each well 250  $\mu$ L of LB Amp/Meth (no glycerol). The original condensed plate was put in storage -80° C. The two condensed daughter plates were incubated at 37° C. for 18 hours.

5 The short chain esterase '600  $\mu$ M substrate stock solution' was prepared as follows: 25 mg of each of the following compounds was dissolved in the appropriate volume of DMSO to yield a 25.2 mM solution. The compounds used were 4-methylumbelliferyl propionate, 4-methylumbelliferyl butyrate, and 4-methylumbelliferyl heptanoate. Two hundred fifty microliters of each DMSO solution was added to ca 9 mL of 50 mM, pH 7.5

Hepes buffer which contained 0.6% of Triton X-100 and 0.6 mg per mL of dodecyl maltoside (Anatrace). The volume was taken to 10.5 mL with the above Hepes buffer to yield a slightly cloudy suspension.

5 The long chain '600  $\mu$ M substrate stock solution' was prepared as follows: 25 mg of each of the following compounds was dissolved in DMSO to 25.2 mM as above. The compounds used were 4-methylumbelliferyl elaidate, 4-methylumbelliferyl palmitate, 4-methylumbelliferyl oleate, and 4-methylumbelliferyl stearate. All required brief warming in a 70° C. bath to achieve dissolution. Two hundred fifty microliters of each DMSO solution was added to the Hepes buffer and diluted to 10.5 mL as above. All seven umbelliferones were  
0 obtained from Sigma Chemical Co.

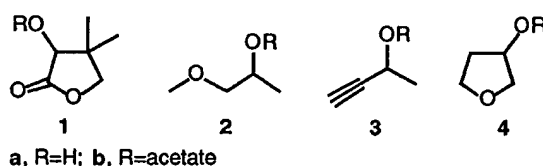
Fifty  $\mu$ L of the long chain esterase or short chain esterase '600  $\mu$ M substrate stock solution' was added to each of the wells of a white condensed plate using the Biomek to yield a final concentration of substrate of about 100  $\mu$ M.. The fluorescence values were recorded (excitation=326 nm, emission=450 nm) on a plate-reading fluorometer immediately after  
5 addition of the substrate. The plate was incubated at 70° C. for 60 minutes in the case of the long chain substrates, and 30 minutes at RT in the case of the short chain substrates. The fluorescence values were recorded again. The initial and final fluorescence values were compared to determine if an active clone was present.

#### **Example 6: Isolation and Purification of the Active Clone**

0 To isolate the individual clone which carried the activity, the Source GeneBank plates were thawed and the individual wells used to singly inoculate a new plate containing LB Amp/Meth. As above, the plate was incubated at 37° C. to grow the cells, 50  $\mu$ L of 600  $\mu$ M substrate stock solution was added using the Biomek and the fluorescence was determined. Once the active well from the source plate was identified, cells from this active well were  
5 streaked on agar with LB/Amp/Meth and grown overnight at 37° C. to obtain single colonies. Eight single colonies were picked with a sterile toothpick and used to singly inoculate the wells of a 96-well microtiter plate. The wells contained 250  $\mu$ L of LB Amp/Meth. The cells were grown overnight at 37° C. without shaking. A 200  $\mu$ L aliquot was removed from each well and assayed with the appropriate long or short chain substrates as above. The most  
3 active clone was identified and the remaining 50  $\mu$ L of culture was used to streak an agar

plate with LB/Amp/Meth. Eight single colonies were picked, grown and assayed as above. The most active clone was used to inoculate 3 mL cultures of LB/Amp/Meth, which were grown overnight. The plasmid DNA was isolated from the cultures and utilized for sequencing.

## 5 Example 7: Synthesis of Ester Substrates



Racemic and optically pure alcohols **1a-4a** were obtained from common suppliers at the highest purity available. Acetates **1b** and **4b** were synthesized from the corresponding acid chloride in pyridine using standard procedures. All organic solvents were analytical grade or higher and dried overnight over activated molecular sieves (3 Å) prior to use.

$^1\text{H}$  NMR und  $^{13}\text{C}$  NMR spectra were recorded at 250/500 and 63/126 MHz, respectively, in  $\text{CDCl}_3$  on Bruker (Karlsruhe, Germany) spectrometers. Chemical shifts ( $\delta$ ) are given in ppm relative to internal TMS, coupling constants ( $J$ ) in Hz. Enantiomeric excess values (% ee) were calculated from gas chromatographic analysis.

### 5 (*R,S*)-2-Acetoxy-3,3-dimethyl- $\gamma$ -butyrolactone (**1b**)

To a solution of (*R,S*)-2-hydroxy-3,3-dimethyl- $\gamma$ -butyrolactone (9.8 g, 75 mmol) in 75 mL pyridine, acetyl chloride (7.5 mL, 105 mmol) was added, while cooling with iced water, and the resultant mixture was stirred at room temperature for 19 hours. Water (50 mL) was then added, and the mixture was extracted with diethyl ether. The organic layer was washed with hydrochloric acid (0.1 mol), saturated sodium carbonate solution, and water. After drying and removal of solvent, the residue was purified by silica gel column chromatography to give 6.4 g (50 %) of pure **1b**:  $^1\text{H}$ -NMR spectra were identical to literature data (Gamalevich and Serebryakov, *Russ. Chem. Bull.* 46: 171 (1997));  $\delta_{\text{C}}$  (126 MHz) 19.89, 20.55, 23.03, 40.16, 75.06, 76.21, 169.79, 172.46; Anal. calcd for  $\text{C}_8\text{H}_{12}\text{O}_4$ : C 55.81, H 7.02. Found: C 55.69, H 7.11.

(R,S)-3-Tetrahydrofuryl-acetate (4b).

To a solution of **4a** (45 mmol; 4.0 g) in 75 mL dichloromethane a five times molar excess of pyridine (16 mL) and acetyl chloride (200 mmol; 14.2 mL) was added, while cooling with iced water. After 24 hours, the reaction mixture was worked-up as described for **1b**, yielding 2.7 g (46 %) of pure **4b**.  $\delta_H$  (500 MHz) 2.02 (1H, m, 4-H), 2.07 (3H, s, COCH<sub>3</sub>), 2.17 (1H, m, 4-H), 3.88 (4H, m, 2-H/5-H), 5.29 (1H, m, 3-H);  $\delta_C$  (126 MHz) 21.13 (CH<sub>3</sub>), 32.69 (4-C), 67.00, 73.14, 74.83 (3-C), 170.91 (CO); Anal. calcd for C<sub>6</sub>H<sub>10</sub>O<sub>3</sub>: C 55.37, H 7.74. Found: C 55.41, H 7.79.

**Example 8: Screening reaction to determine enzymatic activity.**

Screening reactions were performed in 96-well microtiterplates operated by a pipetting robot system (Beckman 2000, Unterschleißheim, Germany). 180  $\mu$ L of sodium phosphate buffer (10 mM; pH 7.3) with an enzyme concentration of ~1 mg hydrolase / mL buffer were mixed with 20  $\mu$ L DMSO containing 20 mM **1b-4b** and bromothymol-blue (0.75 mM). The microtiterplates were covered and incubated at room temperature, 37 °C, 50 °C, or 80 °C for 24 hours. A visually monitored color change indicated active enzymes. Analogous set-ups without substrates or enzymes served as blanks.

**Example 9: Small scale reactions for the determination of enantioselectivities.**

Acetates (**1b-4b**) (50 mM) were added to an enzyme-solution (~1 mg hydrolase / mL) in sodium phosphate buffer (10 mM; pH 7.3). After shaking at room temperature, 37 °C, 50 °C, or 80 °C (reaction times are indicated in Table 1), an aliquot of 500  $\mu$ L was taken, extracted with 250  $\mu$ L dichloromethane (**1b**) or 250  $\mu$ L ethyl acetate (**3b** or **4b**). The organic layer was separated, dried and used directly for GC-analysis after dilution. In the case of substrate **2b**, the aliquot was extracted with 500  $\mu$ L dichloromethane and derivatized with trifluoroacetic acid anhydride prior to gas chromatographic (GC) analysis. The results are shown in Table 1.

**Table 1. Small-scale hydrolyses**

Substrate	Hydrolase	Conditions [°C / h]	Ester [% ee]	Alcohol [% ee]	Conversion [%]	E-value <sup>a</sup>
<b>1b</b>	BD073	80 / 6	68 (R)	94 (S)	42	66



	BD094	rt /	39	49 (R)	>99 (S)	33	>100
	BD405	37 /	17	25 (R)	>99 (S)	20	>100
	BD423	rt /	17	25 (S)	59 (R)	30	5
2b	BD021	37 /	1	27 (R)	79 (S)	26	11
3b	BD021	37 /	26	99 (R)	58 (S)	63	19
	BD213	37 /	26	80 (R)	85 (S)	48	30
4b	BD100	37 /	22	34 (S)	33 (R)	51	3

<sup>a</sup> Calculated according Chen *et al.*, *J. Am. Chem. Soc.* 104: 7294 (1982)

#### Example 10: General procedure for preparative enantioselective hydrolyses.

Preparative reactions were performed similar to the small scale reactions, but in a pH-stat device (Metrohm, Herisau, Switzerland) to maintain the pH at 7.3 and monitor the conversion by addition of 0.1 or 1.0 N NaOH solution. Enzyme amounts, substrate concentrations and reaction temperatures are indicated in Table 2. After the desired conversion had been achieved, the reaction was stopped by extraction with diethyl ether. The combined organic layers were dried and the solvent was removed by evaporation in vacuum. Alcohol and non-reacted ester were separated by silica gel column chromatography. Enantiomeric excess and conversion were determined by GC analysis, and chemical identity was confirmed by NMR spectroscopy. The results are shown in Table 2.

**Table 2. Preparative-scale hydrolyses**

Substr.	Hydrolase	Cond.	Conversion	Ester		Alcohol		E-value <sup>a</sup>
[mmol]	[mg]	[°C / min]	[%] <sup>a</sup>	yield [%]	[% ee]	yield [%]	[% ee]	
1b / 2	BD423 / 15	rt / 9	75	21	>99 (S)	27	28 (R)	5
2b / 20	BD021 / 20	37 / 383	7	n.d.	3 (R)	n.d.	28 (S)	2
4b / 2	BD100 / 15	80 / 123	70	4	49 (S)	n.d.	24 (R)	3

<sup>a</sup> Calculated according Chen *et al.*, *J. Am. Chem. Soc.* 104: 7294 (1982)

While the foregoing invention has been described in some detail for purposes of clarity and understanding, these particular embodiments are to be considered as illustrative and not restrictive. It will be appreciated by one skilled in the art from a reading of this

disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

## WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:1 and sequences having at least about 50% identity to SEQ ID NO:1.
- 5 2. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:3 and sequences having at least about 50% identity to SEQ ID NO:3.
3. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:5  
) and sequences having at least about 60% identity to SEQ ID NO:5.
4. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:7 and sequences having at least about 60% identity to SEQ ID NO:7.
- 5 5. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:9  
) and sequences having at least about 90% identity to SEQ ID NO:9.
6. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:11 and sequences having at least about 50% identity to SEQ ID NO:11.
- ) 7. An isolated nucleic acid encoding a polypeptide having hydrolase activity, said nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:13 and sequences having at least about 50% identity to SEQ ID NO:13.
8. An isolated nucleic acid complementary to the nucleic acid of any one of claims 1-7.

9. An isolated nucleic acid that hybridizes under conditions of high stringency to a nucleic acid having a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13.
- 5 10. An isolated nucleic acid that hybridizes under conditions of moderate stringency to a nucleic acid having a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13.
11. An isolated nucleic acid that hybridizes under conditions of low stringency to a nucleic acid having a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, and 13.
12. An isolated nucleic acid having a sequence with at least about 55% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
13. An isolated nucleic acid having a sequence with at least about 60% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
- 5 14. An isolated nucleic acid having a sequence with at least about 65% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
15. An isolated nucleic acid having a sequence with at least about 70% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
16. An isolated nucleic acid having a sequence with at least about 75% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.

17. An isolated nucleic acid having a sequence with at least about 80% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
18. An isolated nucleic acid having a sequence with at least about 85% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
19. An isolated nucleic acid having a sequence with at least about 90% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
20. An isolated nucleic acid having a sequence with at least about 95% homology to the nucleic acid set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, or 13, as determined with a sequence comparison algorithm.
21. The isolated nucleic acid of any one of claims 12-20, wherein the sequence comparison algorithm is FASTA version 3.0t78 with the default parameters.
22. An isolated nucleic acid comprising at least 10 consecutive bases of a sequence selected from the group consisting of SEQ ID NOS:1, 3, 5, 7, 9, 11, and 13, and sequences complementary thereto.
23. A purified polypeptide comprising an amino acid sequence with at least about 50% identity to the amino acid sequence set forth in SEQ ID NO:2.
24. A purified polypeptide comprising an amino acid sequence with at least about 50% identity to the amino acid sequence set forth in SEQ ID NO:4.
25. A purified polypeptide comprising an amino acid sequence with at least about 55% identity to the amino acid sequence set forth in SEQ ID NO:6.

26. A purified polypeptide comprising an amino acid sequence with at least about 55% identity to the amino acid sequence set forth in SEQ ID NO:8.

27. A purified polypeptide comprising an amino acid sequence with at least about 99% identity to the amino acid sequence set forth in SEQ ID NO:10.

5 28. A purified polypeptide comprising an amino acid sequence with at least about 50% identity to the amino acid sequence set forth in SEQ ID NO:12.

29. A purified polypeptide comprising an amino acid sequence with at least about 50% identity to the amino acid sequence set forth in SEQ ID NO:14.

0 30. A purified polypeptide comprising an amino acid sequence with at least about 50% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

5 31. A purified polypeptide comprising an amino acid sequence with at least about 55% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

0 32. A purified polypeptide comprising an amino acid sequence with at least about 60% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

33. A purified polypeptide comprising an amino acid sequence with at least about 65% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

34. A purified polypeptide comprising an amino acid sequence with at least about 70% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

5 35. A purified polypeptide comprising an amino acid sequence with at least about 75% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

0 36. A purified polypeptide comprising an amino acid sequence with at least about 80% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

5 37. A purified polypeptide comprising an amino acid sequence with at least about 85% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

0 38. A purified polypeptide comprising an amino acid sequence with at least about 90% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

39. A purified polypeptide comprising an amino acid sequence with at least about 95% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 12, or 14, as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

5 40. A purified polypeptide comprising an amino acid sequence with at least about 99% homology to the amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, or 14,

as determined by analysis with a sequence comparison algorithm or FASTA version 3.0t78 with the default parameters.

41. A purified polypeptide comprising a sequence selected from the group consisting of SEQ ID NOS:2, 4, 6, 8, 10, 12, and 14.

5 42. An isolated nucleic acid encoding a polypeptide according to any one of claims 23-29.

43. An isolated nucleic acid encoding a polypeptide comprising at least 10 consecutive amino acids of a polypeptide according to any one of claims 23-29.

) 44. A purified antibody that specifically binds to a polypeptide according to any one of claims 23-29.

45. A purified antibody that specifically binds to a polypeptide having at least 10 consecutive amino acids of the polypeptide according to any one of claims 23-29.

46. The antibody of claim 44, wherein the antibodies are polyclonal.

47. The antibody of claim 45, wherein the antibodies are polyclonal.

5 48. The antibody of claim 44, wherein the antibodies are monoclonal.

49. The antibody of claim 45, wherein the antibodies are monoclonal.

50. A method of producing the polypeptide according to any one of claims 23-29, comprising introducing a nucleic acid encoding the polypeptide into a host cell under conditions that allow expression of the polypeptide and recovering the polypeptide.

) 51. A method of producing a polypeptide comprising at least 10 amino acids of a peptide according to any one of claims 23-29, comprising introducing a nucleic acid encoding



the polypeptide, operably linked to a promoter, into a host cell under conditions that allow expression of the polypeptide and recovering the polypeptide.

52. A method of generating a variant comprising:

- (a) obtaining a nucleic acid according to any one of claims 1-7; and
- 5 (b) modifying one or more nucleotides in said sequence to another nucleotide, deleting one or more nucleotides in said sequence, or adding one or more nucleotides to said sequence.

53. The method of claim 52, wherein the modifications are introduced by a method selected from the group consisting of error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-specific mutagenesis, gene reassembly, gene site saturated mutagenesis, and any combination thereof.

54. A computer readable medium having stored thereon the nucleic acid sequence of a nucleic acid according to any one of claims 1-7 or the amino acid sequence of a polypeptide according to any one of claims 23-29.

55. A computer system comprising a processor and a data storage device, wherein said data storage device has stored thereon the nucleic acid sequence of a nucleic acid according to any one of claims 1-7 or the amino acid sequence of a polypeptide according to any one of claims 23-29.

56. The computer system of claim 55, further comprising a sequence comparison algorithm and a data storage device having at least one reference sequence stored thereon.

57. The computer system of claim 56, wherein the sequence comparison algorithm comprises a computer program which indicates polymorphisms.

58. The computer system of claim 55, further comprising an identifier which identifies features in said sequence.

59. A method for comparing a first sequence to a reference sequence, comprising:

5 (a) reading the first sequence and the reference sequence through use of a computer program which compares sequences; and

(b) determining differences between the first sequence and the reference sequence with the computer program;

wherein said first sequence is the sequence of a nucleic acid according to any one of claims 1-7 or the amino acid sequence of a polypeptide according to any one of claims 23-29.

0 60. The method of claim 59, wherein determining differences between the first sequence and the reference sequence comprises identifying polymorphisms.

61. A method for identifying a feature in a sequence, comprising:

(a) reading the sequence through the use of a computer program which identifies features in sequences; and

5 (b) identifying features in the sequences with the computer program;

wherein said sequence is the sequence of a nucleic acid according to any one of claims 1-7 or the amino acid sequence of a polypeptide according to any one of claims 23-29.

62. A purified polypeptide according to any one of claims 23-29, wherein the polypeptide is an enzyme which is stable to heat, is heat resistant, and catalyzes the  
0 hydrolysis of esters, and wherein the enzyme is able to renature and regain activity after exposure to temperatures of from about 60 degrees C to 105 degrees C.

63. A method of catalyzing the hydrolysis of an ester comprising contacting a sample containing an ester with a polypeptide according to any one of claims 23-29.

64. An assay for identifying a functional polypeptide encoded by the nucleic acid according to claim 43 that retains hydrolase activity, said assay comprising:

- 5 (a) contacting a polypeptide encoded by the nucleic acid according to claim 43 with a substrate molecule under conditions which allow said polypeptide or fragment or variant to function; and
- (b) detecting either a decrease in the level of substrate or an increase in the level of the specific reaction product of the reaction between said polypeptide and substrate, wherein a decrease in the level of substrate or an increase in the level of the reaction product is indicative of a functional polypeptide or fragment or variant.

0 65. A nucleic acid probe comprising a nucleic acid according to claim 22, which probe hybridizes to the nucleic acid target region of a nucleic acid sequence selected from the group consisting of SEQ ID NOS:1, 3, 5, 7, 9, 11, and 13 under moderate to highly stringent conditions to form a detectable target:probe duplex.

66. The probe of claim 65, wherein the nucleic acid is DNA.

5 67. The probe of claim 65, wherein the nucleic acid has a sequence selected from the group consisting of nucleic acid sequences that are at least 55% complementary to the nucleic acid target region, nucleic acid sequences that are at least 60% complementary to the nucleic acid target region, nucleic acid sequences that are at least 65% complementary to the nucleic acid target region, nucleic acid sequences that are at least 70% complementary to the nucleic acid target region, nucleic acid sequences that are at least 75% complementary to the nucleic acid target region, nucleic acid sequences that are at least 80% complementary to the nucleic acid target region, nucleic acid sequences that are at least 85% complementary to the nucleic acid target region, nucleic acid sequences that are at least 90% complementary to the nucleic acid target region, nucleic acid sequences that are at least 95% complementary to the nucleic acid target region, and nucleic acid sequences that are at least 99% complementary to the nucleic acid target region.

0

5

68. The probe of claim 65, wherein the nucleic acid is fully complementary to the nucleic acid target region.

69. The probe of claim 65, wherein the oligonucleotide is 15-50 bases in length.

70. The probe of claim 65, wherein the probe further comprises a detectable isotopic label.

71. The probe of claim 65, wherein the probe further comprises a detectable non-isotopic label selected from the group consisting of a fluorescent molecule, a chemiluminescent molecule, an enzyme, a cofactor, an enzyme substrate, and a hapten.

72. An enzyme preparation comprising a polypeptide of any one of claims 23-29, wherein the preparation is liquid.

73. An enzyme preparation comprising a polypeptide of any one of claims 23-29, wherein the preparation is dry.

74. A process for resolving an enantiomeric mixture of esters, comprising:

(a) contacting the ester mixture with a polypeptide according to any one of claims 23-29 to hydrolyze the ester to an alcohol; and

(b) recovering (i) a mixture of esters enriched in one enantiomer and/or (ii) a mixture of alcohols enriched in the opposite enantiomer.

75. The process of claim 74, wherein the ester is a C<sub>1</sub>-C<sub>6</sub> alkanoate ester of a secondary alcohol.

76. The process of claim 75, wherein the ester is an acetate.

77. The process of claim 75, wherein the secondary alcohol is selected from the group consisting of 2-hydroxy-3,3-dimethyl- $\gamma$ -butyrolactone, 3-butyne-2-ol, 1-methoxy-2-propanol, and 3-hydroxytetrahydrofuran.

78. A process for resolving an enantiomeric mixture of alcohols, comprising:

(a) contacting the alcohol mixture with an acyl donor and a polypeptide according to any one of claims 23-29 to esterify the alcohol; and

5 (b) recovering (i) a mixture of esters enriched in one enantiomer and/or (ii) a mixture of alcohols enriched in the opposite enantiomer.

79. The process of claim 78, wherein the secondary alcohol is selected from the group consisting of 2-hydroxy-3,3-dimethyl- $\gamma$ -butyrolactone, 3-butyne-2-ol, 1-methoxy-2-propanol, and 3-hydroxytetrahydrofuran.

80. The process of claim 78, wherein the acyl donor is vinyl acetate.

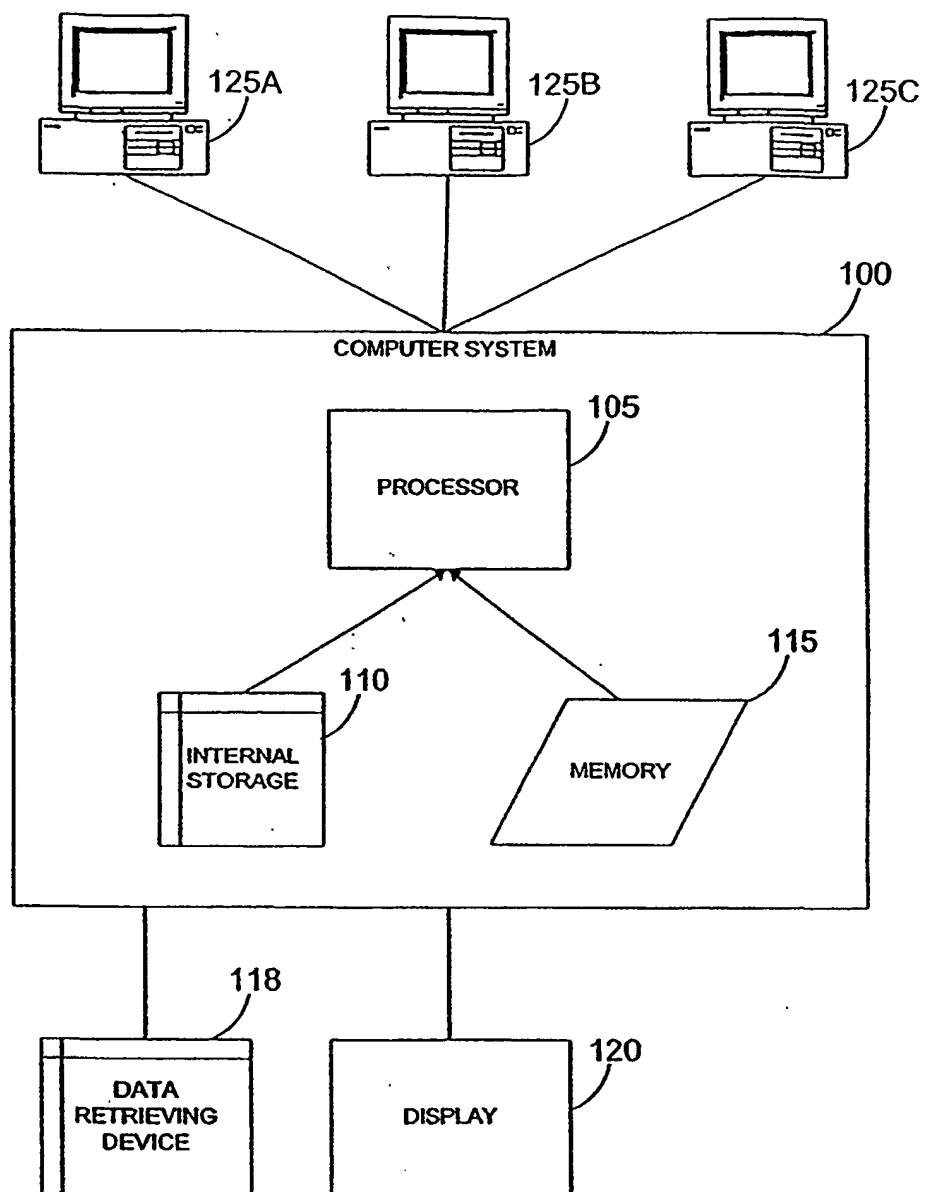


FIGURE 1

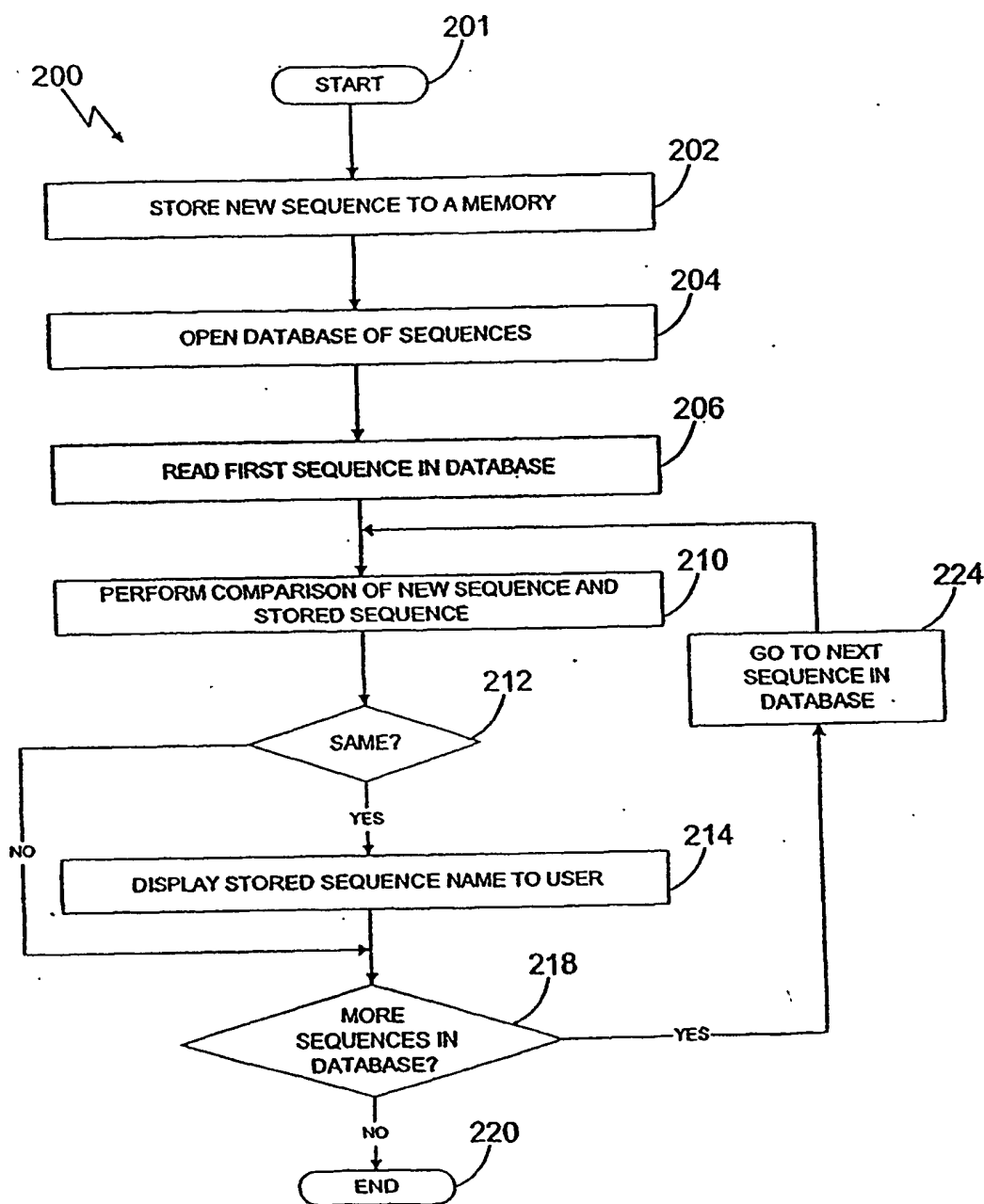
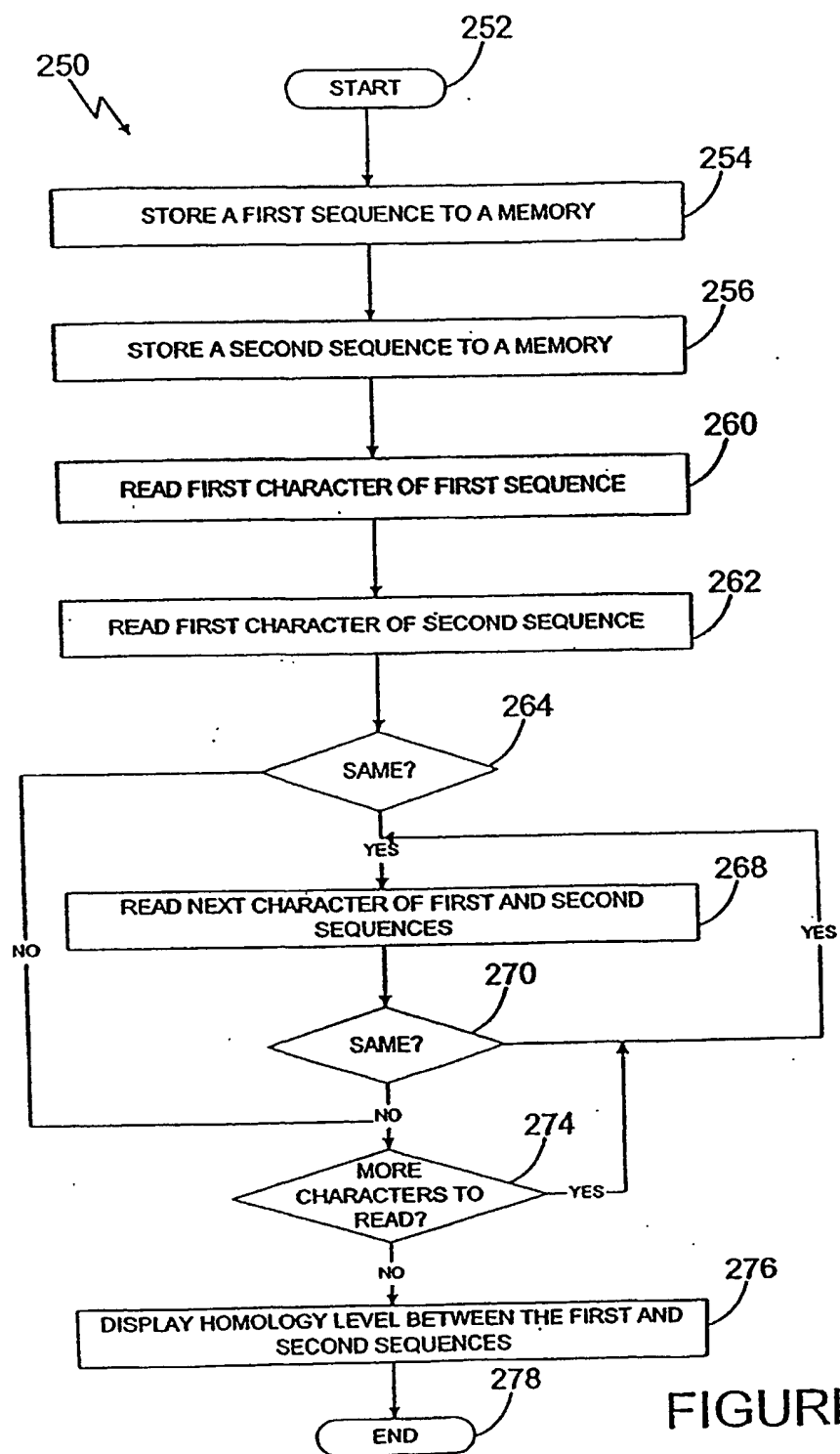


FIGURE 2





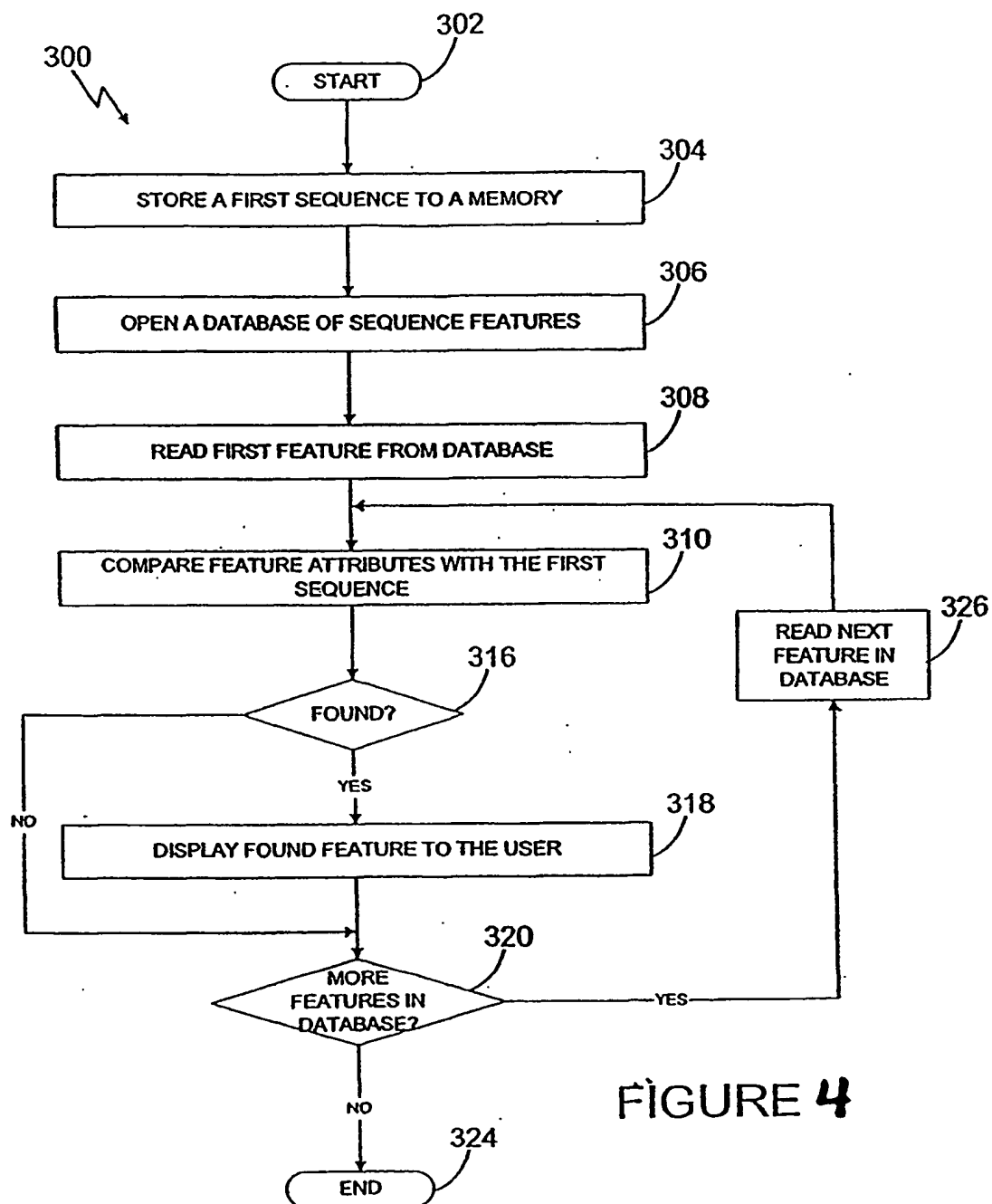


FIGURE 4

**Figure 5A**

SEQ ID NO:1 BD021

gtgagcattcgtctcgactgttaaaactggttttgaatacctttgaaaaaaccaaaactggccgcggccaaaacgccggatgatttgcgaaaatc  
 gtttgaattaaaggcaggttttggttccggcgccacgtaaaacaaggtttagtcatgatgtattgcagtcagggcatcgggtcgggtataatgccc  
 agtgggcgaaatccaaatctgcatctgatgacagggtaatcctgtatttcacgggggaggggtatgttttgggtcaccaaaaacgacccgtgc  
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 gcttgccttgagaaaatcaaggcccaatccttgcccaaacctgctggcggttttgccctgtcgccttgggttgatttatcatttccggcccttcgtttt  
 ctaaaaatgcccaaccgatgtgatgttggcccgcatcacgggctgcgggatatggcgacctgtatttggatggggccgatgcagatgatccac  
 gtgcatcggccgtgcaggcggttttctggcatgcccgccgtatttctgacaggcaagtgcacagtgaatacctgttggatgattgcctggcgatg  
 gcggatcacttgcgtgcgcaagggtgtcgttggacagaccggattgttgaaaaccatccacatgttggcataatttttcaacgccttctaccggaa  
 gcagatcaggggctgcgggcgattgccgcgtgggattaaacctcttttatacagggttcaaacgaaagctaa

**Figure 5B**

SEQ ID NO.2 BD021

Val Ser Ile Arg Leu Arg Leu Asn Trp Phe Leu Asn Thr Phe Glu Lys Pro Lys Leu Ala Ala Lys Thr Pro  
 Asp Asp Leu Arg Lys Ser Phe Glu Leu Lys Ala Arg Phe Leu Phe Pro Ala Pro Arg Lys Thr Arg Phe Ser His  
 Asp Val Leu Gln Ser Gly Ile Gly Ser Val Asn Ala Gln Trp Ala Lys Ser Lys Ser Ala Ser Asp Arg Val Ile  
 Leu Tyr Phe His Gly Gly Tyr Val Phe Gly Ser Pro Lys Thr His Arg Ala Met Leu Ala Arg Leu Ser Ala Met  
 Thr Gly Leu Ser Ala Cys Leu Pro Asp Tyr Arg Leu Ala Pro Glu His Pro Phe Pro Ala Ala Ile Glu Asp Ala Val  
 Leu Ser Tyr Lys Cys Leu Leu Glu Arg Ala Ile Glu Pro Gln Asn Ile Ile Leu Gly Gly Asp Ser Ala Gly Gly Gly  
 Leu Val Leu Ala Leu Ala Glu Ile Lys Ala Gln Ser Leu Pro Lys Pro Ala Gly Val Phe Ala Leu Ser Pro Leu  
 Val Asp Leu Ser Phe Ser Gly Leu Ser Phe Ser Lys Asn Ala Gln Thr Asp Val Met Leu Pro Ala Ser Arg Ala Ala  
 Asp Met Ala Thr Leu Tyr Leu Asp Gly Ala Asp Ala Asp Pro Arg Ala Ser Pro Leu Gln Ala Asp Phe Ser  
 Gly Met Pro Pro Val Phe Leu Thr Ala Ser Asp Ser Glu Ile Leu Leu Asp Asp Cys Leu Arg Met Ala Asp His Leu  
 Arg Ala Gln Gly Val Val Thr Asp Arg Ile Val Glu Asn His Pro His Val Trp His Ile Phe Gln Arg Leu Leu  
 Pro Glu Ala Asp Gln Gly Leu Arg Ala Ile Ala Trp Ile Lys Pro Leu Leu Ser Gly Ser Asn Glu Ser

Figure 6A

SEQ ID NO:3 BD213

atgttacgcacactttaccctgatcttgggtattgatggggcttttcccatgtcctctacgggttatggccgatacggccaaaacttccactatttttagtg  
 tggggcgacagttaaagtgcggccttatggcatcccggtcgaaaaaggctgggtcaatttaatgcgaaaccaagfttaggcactgagtttaatgtcg  
 tcaatggcagtattagtgagaaaccactgctggaggcttaagccgcttacctaaaggcattaaacagattatcaacctgattatgtattgatagaat  
 tagggcggaatgatgggttgcatgggtattgtctaccgacatcatgaaaggctaaaccttagcaaaatgattgaaactcagtcagaccaatcatgcccaa  
 ggtgtgattaattgggattcagctcccccaatttcggtaatgctttttacggataagtttgatgccacttatcaggatttagctaaacaataaactt  
 gcccttagtgccatcataatgaccaatgtggcagaaaaattgggatttaattcaagctgatggtttgcaccaaccgcaggaagcacagccattatt  
 gcttgaaaaatgtatggaagggtgtagaaaccggttttaaaaccaactactccccgccaaatcataa

**Figure 6B**

SEQ ID NO:4 BD213

Met Leu Arg Thr Leu Tyr Leu Ile Leu Val Leu Met Gly Leu Phe Pro Leu Ser Ser Thr Val Met  
 Ala Asp Thr Pro Lys Thr Ser Thr Ile Leu Val Trp Gly Asp Ser Leu Ser Ala Ala Tyr Gly Ile Pro  
 Val Glu Lys Gly Trp Val Asn Leu Met Arg Thr Lys Leu Gly Thr Glu Phe Asn Val Val Asn Gly  
 Ser Ile Ser Gly Glu Thr Thr Ala Gly Gly Leu Ser Arg Leu Pro Lys Ala Leu Thr Asp Tyr Gln Pro  
 Asp Tyr Val Leu Ile Glu Leu Gly Ala Asn Asp Gly Leu His Gly Ile Ala Thr Asp Ile Met Lys Ala  
 Asn Leu Ser Lys Met Ile Glu Leu Ser Gln Thr Asn His Ala Lys Val Val Leu Ile Gly Ile Gln Leu  
 Pro Pro Asn Phe Gly Asn Ala Phe Thr Asp Lys Phe Asp Ala Thr Tyr Thr Asp Leu Ala Lys Gln  
 Tyr Asn Leu Pro Leu Val Pro Ser Leu Met Thr Asn Val Ala Glu Asn Trp Asp Leu Ile Gln Ala  
 Asp Gly Leu His Pro Thr Ala Glu Ala Gln Pro Leu Leu Glu Asn Val Trp Lys Val Val Glu  
 Thr Val Leu Lys Pro Thr Thr Pro Ala Lys Ser

Figure 7A

SEQ ID NO:5 BD405

atgaaaattaagtgccagaaccattcacattcgaagcaggaaatcggcaggtttattattacatggatttactggccattcagc  
agatgtaagaatgcttggtcgtgattctagaaaaaggttatcaactcatgaccaatttatagaggcatggtcaagagccc  
agaaagcattattaaaatcttcacctgatgaatgggggaggtattttatcagcttataatcatttgagaaatctaggtatataatga  
aattgctgttgctgttccaatgggtggctttagcaattaaactagctaccaagcatgaaataaaagggtgtcattccaatgt  
gtacaccaatgtactttgataatacaaaaaacaattaactaaagcatttttaaacttgcacgacaatttaaaaaaattgagaaaaag  
atgaacaaacaattaataaaagaaatagatttactacaacaaaattcagcagaattatttaatagaaatcggcactttttagaaca  
gtcaatggataatagatagagttgacgtacctacattcgtagtccaagcaagtaaagatgaaataataaatccctgaaaagtgcg  
acatttattatgaaaacattaaaaatgaaaaaaagatttaaaatggtataaaaactcaactcacttgataacattggcgcgatgaa  
aaagatgttttacatgaaagatatatatcatttttttagaaacattagattggaacaactaa

**Figure 7B**

SEQ ID NO:6 BD405

Met Lys Ile Lys Leu Pro Glu Pro Phe Thr Phe Glu Ala Gly Asn Arg Ala Val Leu Leu Leu His  
 Gly Phe Thr Gly His Ser Ala Asp Val Arg Met Leu Gly Arg Phe Leu Glu Lys Lys Gly Tyr Thr  
 Thr His Ala Pro Ile Tyr Arg Gly His Gly Gln Glu Pro Glu Ala Leu Leu Lys Ser Ser Pro Asp Glu  
 Trp Trp Glu Asp Ile Leu Ser Ala Tyr Asn His Leu Arg Asn Leu Gly Tyr Asn Glu Ile Ala Val Ala  
 Gly Leu Ser Met Gly Gly Ala Leu Ala Ile Lys Leu Ala Thr Lys His Glu Ile Lys Gly Val Ile Pro  
 Met Cys Thr Pro Met Tyr Phe Asp Asn Gln Lys Gln Leu Thr Lys Ala Phe Leu Asn Phe Ala Arg  
 Gln Phe Lys Lys Phe Glu Lys Lys Asp Glu Gln Thr Ile Lys Lys Glu Ile Asp Leu Leu Gln Gln  
 Asn Ser Ala Glu Leu Phe Asn Glu Ile Gly Thr Phe Val Glu Gln Val Asn Gly Ile Ile Asp Arg Val  
 Asp Val Pro Thr Phe Val Val Gln Ala Ser Lys Asp Glu Ile Ile Asn Pro Glu Ser Ala Thr Phe Ile  
 Tyr Glu Asn Ile Lys Asn Glu Lys Lys Asp Leu Lys Trp Tyr Lys Asn Ser Thr His Leu Ile Thr Phe  
 Gly Asp Glu Lys Asp Val Leu His Glu Asp Ile Tyr His Phe Leu Glu Thr Leu Asp Trp Asn Asn

Figure 8A

SEQ ID NO:7 BD100

atgccccctacatccagaggtaaagaatttctccagctacctccccagggtctctccagaaacgtgcaaggagctgagggaaaggccctgggga  
 tttagccctctcagggagggagagagccctgggagaggggttagagaccttgagataccacctagggacgcacgaatcagggccaggggtctcta  
 caccctccaaagtaaggaaaacttaccctgctctgtttactatcacggcgggtgcttcgtgttcggtagcgttgacagctacgacggcctcgcga  
 tcccttiattgccaaagggaatctggggattgctgggttatctcctggtgagtatagggctcgcctccctgagcacaaagtctcccaaccggcagtcacacgactcgt  
 gggatgctgctctctggatcgcggagaaaggagggcaagctggggctcgcacacctcgagacttgccctggctgggggatagtgctgggagggaa  
 acctgtctgcctggtgtccctcctggacagggaccagggtaaggagctgggttagttatcagggtcctaatactaccagcagtgaaacatgggtcgc  
 ataactcccacatccgtcaggggaggtacggcgaggagatacttctcaccaggtccatgatgaactgggttcgggaccatgtacttctctctggaaag  
 ggaaagggtatccccctacgcctctccagccttggtgacctacataaccctccacccctcactggtgtagtcactgcagagtagtatccccccaaag  
 ggatcaggggagagacctactctcactctccctaaacgaggctggaaacgtatatcaaaccttggttagatatcaagggaatgattcacggcttctctgtcc  
 ttctacgagtgagataactgccggtaaacctagccattcaccacattgctgggggttctgagatctgtcctttag



**Figure 8B**

SEQ ID NO:8 BD100

Met Pro Leu His Pro Glu Val Lys Lys Leu Ser Gln Leu Pro Pro Gln Gly Phe Ser Arg Asn  
 Val Gln Glu Leu Arg Lys Ala Trp Asp Leu Ala Phe Ser Gly Arg Arg Glu Ser Leu Glu Arg Val  
 Glu Asp Leu Glu Ile Pro Thr Arg Asp Ala Arg Ile Arg Ala Arg Val Tyr Thr Pro Ser Ser Lys Glu  
 Asn Leu Pro Val Leu Val Tyr Tyr His Gly Gly Phe Val Phe Gly Ser Val Asp Ser Tyr Asp  
 Gly Leu Ala Ser Leu Ile Ala Lys Glu Ser Gly Ile Ala Val Ile Ser Val Glu Tyr Arg Leu Ala Pro  
 Glu His Lys Phe Pro Thr Ala Val Asn Asp Ser Trp Asp Ala Leu Leu Trp Ile Ala Glu Asn Gly Gly  
 Lys Leu Gly Leu Asp Thr Ser Arg Leu Ala Val Ala Gly Asp Ser Ala Gly Gly Asn Leu Ser Ala  
 Val Val Ser Leu Leu Asp Arg Asp Gln Gly Lys Gly Leu Val Ser Tyr Gln Val Leu Ile Tyr Pro Ala  
 Val Asn Met Val Asp Asn Ser Pro Ser Val Arg Glu Tyr Gly Glu Gly Tyr Phe Leu Thr Arg Ser  
 Met Met Asn Trp Phe Gly Thr Met Tyr Phe Ser Ser Gly Arg Glu Ala Val Ser Pro Tyr Ala Ser Pro  
 Ala Leu Ala Asp Leu His Asn Leu Pro Pro Ser Leu Val Ile Thr Ala Glu Tyr Asp Pro Leu Arg Asp  
 Gln Gly Glu Thr Tyr Ser His Ser Leu Asn Glu Ala Gly Asn Val Ser Thr Leu Val Arg Tyr Gln Gly  
 Met Ile His Gly Phe Leu Ser Phe Tyr Glu Trp Ile Thr Ala Gly Lys Leu Ala Ile His His Ile Ala Gly  
 Val Leu Arg Ser Val Leu

**Figure 9A**

SEQ ID NO:9 BD073

atgccgctcgtatcccgtcattcagcagggtgctcgtatcaactcaaccggcatggcctgccccggactacaaacatctctccgccccagcaatttcgtt  
 cccaacagtcgctgttctcctctgtcaagaaaggagccccgtggccgaggtccgagaggttgacatgcatctgcctggcccgccacgctcaagggtg  
 cgcattgtacccggagggcggtcgaaccgcctacccccgcgtcgtgtattatcacggcggttggtgggtcgtcggagagaccctcggagacg  
 cagcatcccgcttgccgcgtcctcgcgaaagacggccgcgcgtcgtgtgttctccgtcgtactaccgctggcgccggagagcacaaagtcccttg  
 ccggccgtggaaagacggccttacgacggcgttcagtggtgacggcgagcgcgagcggactttcatctcgtatccagccccgcacgcggctcggcg  
 gagacagcgccggaggggaatcttgccgctgtgacgagcatccttgcccaaaagagcgcgcgccgcacgcgttccagcgtgctcatct  
 acccttcacgggggtacgacgccggctcctcccgcatctatcgaaagaaaatgaggaaaggctatctcgtgaccggcgcatgatgctctgggt  
 tccgggataatacttgaacagccctggaggaaactcagcatccgtgggttttcacccgtcctctacccggacttgagcgggttgcctccggcggtg  
 catcggcgacggcgacgtacgatccgctgcgcgacgtcggcaagcttttacgcggaaagcgctgaacaaaggcggtcgaagggtcggagatcgg  
 agaaacttcgaaagatctgatccacgggttcgcacagttttacagcccttcggccgtggcgacgaaaggcgctcgtccgcatctggcggaagaaacttc  
 gagagcgcgctggccctga

**Figure 9B**

SEQ ID NO:10 BD073

Met Pro Leu Asp Pro Val Ile Gln Gln Val Leu Asp Gln Leu Asn Arg Met Pro Ala Pro Asp Tyr  
 Lys His Leu Ser Ala Gln Gln Phe Arg Ser Gln Gln Ser Leu Phe Pro Pro Val Lys Lys Glu Pro Val  
 Ala Glu Val Arg Glu Phe Asp Met Asp Leu Pro Gly Arg Thr Leu Lys Val Arg Met Tyr Arg Pro  
 Glu Gly Val Glu Pro Pro Tyr Pro Ala Leu Val Tyr Tyr His Gly Gly Gly Trp Val Val Gly Asp Leu  
 Glu Thr His Asp Pro Val Cys Arg Val Leu Ala Lys Asp Gly Arg Ala Val Val Phe Ser Val Asp  
 Tyr Arg Leu Ala Pro Glu His Lys Phe Pro Ala Ala Val Glu Asp Ala Tyr Asp Ala Leu Gln Trp Ile  
 Ala Glu Arg Ala Ala Asp Phe His Leu Asp Pro Ala Arg Ile Ala Val Gly Gly Asp Ser Ala Gly Gly  
 Asn Leu Ala Ala Val Thr Ser Ile Leu Ala Lys Glu Arg Gly Gly Pro Ala Ile Ala Phe Gln Leu Leu  
 Ile Tyr Pro Ser Thr Gly Tyr Asp Pro Ala His Pro Pro Ala Ser Ile Glu Glu Asn Ala Glu Gly Tyr  
 Leu Leu Thr Gly Gly Met Met Leu Trp Phe Arg Asp Gln Tyr Leu Asn Ser Leu Glu Glu Thr  
 His Pro Trp Phe Ser Pro Val Leu Tyr Pro Asp Leu Ser Gly Leu Pro Pro Ala Tyr Ile Ala Thr Ala  
 Gln Tyr Asp Pro Leu Arg Asp Val Gly Lys Leu Tyr Ala Glu Ala Leu Asn Lys Ala Gly Val Lys  
 Val Glu Ile Glu Asn Phe Glu Asp Leu Ile His Gly Phe Ala Gln Phe Tyr Ser Leu Ser Pro Gly Ala  
 Thr Lys Ala Leu Val Arg Ile Ala Glu Lys Leu Arg Asp Ala Leu Ala

**Figure 10A**

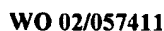
SEQ ID NO:11 BD094

atgaaaaagataattatatatactcttaggattggttctgtctacttgattgttagccctgtgaacctgaattttcatcacctgttgatgaaagtaattatacgaagcgggtgaaagc  
 tgatttttagcaaatatgtatgctataggtaattcgccttctgcccggatttaccaatggcaccttgtataaaagcgggacaagaaaaactttttcttccatgctgggccaaccaaa  
 atgaaaacttgcaagcgggtggaatttaccacaacctatatatgccgatgacaccaatgatgtgggtgtatgaaaaatgggtatgcttaaccatcctagctccttaaattha  
 gttatcgaatgcttctgaaaggaaagaccctgaacgcatacaatgaaaactccttcaatggatgtgataatcctccggcccataataacaatgggtgtacccctggccc  
 aaaatatctcatgttgtagctcccggctatggtaatgcagcccaattggcctgggactagccaatccgtattttgtaaaggatggcttctgctcccgaacaaaacaggtatt  
 ggaaagacatcatgggtcaacaacactacttcttaccctttggatcggaaataaacgatgtactatggtactaccgggtggaggaaccggtacagatcaagacgaaag  
 ctggaaaacttggatcccagttacttatgtctcgggaagatttaacccaatgccaatgtattcggacaatactatatagtaataatgggttaaccgtctttacagcccaacggagccaa  
 agggagtagtagctaccataacctgatgtagcatctgtaccctttttacaactatcccttaccgtctccgcttgaccccggcaggtaatgaaaacatatggccaagtatgattccaac  
 acttaaatgctcaatacggcctgtgtaaatcaaggccctttacagctctgggtgttccggaaaaggcaattgttttctccgaaagacaaccccagtcctcttggttaatttttgataaa  
 gatttaaacgggatattcccgcctcaattaacagcctggccttgcaagcagggtggatttagatttaccacaacagctactctattgggtacacacaattcggacaatggccgtcaagccca  
 ctgaaaaatgatttagtcttattaaaccgcaaaatcagtaatacggtaaaagttaaaggtaacttcacagagatgctcttgttaaataatgggtgaggttctctatttagatggccactcaatt  
 atctattgaagggtattaccctatcctatagaagaccaatgggattttaaccgaaaatgaaaaccggacatgtaagaaaacgtaaacagccaaaactcaacggggtttatcactgct  
 attggcggatggccaatgataatgtttagtagccgatatggctgctgttatgcaagatatgtcaaacgggactgattgttagaaagacgggttctatctataccgcagattattttt  
 aacgggaaaaaatctgggatgaattaaagtcttggccttggacgggggtacacccctactccccgtggatatgccattatagctaaatgaatttattcatgttatcgaaaaaaactt  
 cgggtgctaaattaccctaaattatccggctcaatatcctactttttagattttttatccagtaactaa

**Figure 10B**

SEQ ID NO.12 BD094

Met Lys Lys Ile Leu Tyr Ile Leu Leu Gly Leu Val Leu Ser Thr Gly Phe Val Ala Cys Glu Pro Glu Phe Ser Ser  
Pro Val Asp Glu Ser Asn Tyr Thr Ser Gly Glu Ala Asp Phe Ser Lys Tyr Val Ala Ile Gly Asn Ser Leu Ser Ala  
Gly Phe Thr Asn Gly Thr Leu Tyr Lys Ser Gly Gln Glu Asn Ser Phe Pro Ser Met Leu Ala Asn Gln Met Lys  
Leu Ala Gly Gly Glu Phe Thr Gln Pro Tyr Met Gly Asp Asp Thr Asn Asp Val Gly Gly Met Lys Met Gly  
Ser Leu Thr Ile Leu Ala Pro Lys Leu Val Ile Asp Ala Ser Glu Gly Arg Pro Glu Arg Ile Asn Glu Thr Pro Ser  
Met Asp Val Met Asn Ile His Pro Gly Pro Tyr Asn Asn Met Gly Val Pro Ala Ala Lys Ile Phe His Leu Val Ala  
Pro Gly Tyr Gly Asn Ala Ala Asn Leu Pro Ala Gly Leu Ala Asn Pro Tyr Phe Val Arg Met Ala Ser Ala Pro Asp  
Lys Thr Val Leu Glu Asp Ile Met Ala Gln Gln Pro Thr Phe Phe Thr Leu Trp Ile Gly Asn Asn Asp Val Leu Trp  
Tyr Ala Thr Gly Gly Thr Gly Thr Asp Gln Asp Glu Ala Gly Asn Leu Asp Pro Ser Thr Tyr Gly Pro Glu Asp  
Leu Thr Asn Ala Asn Val Phe Gly Gln Ile Tyr Ser Asn Met Val Thr Ala Leu Thr Ala Asn Gly Ala Lys Gly Val  
Val Ala Thr Ile Pro Asp Val Ala Ser Val Pro Phe Thr Thr Ile Pro Tyr Ala Pro Leu Asp Pro Ala Ser Asn Glu  
Thr Tyr Ala Ser Met Ile Pro Thr Leu Asn Ala Gln Tyr Gly Leu Leu Asn Gln Ala Phe Thr Ala Leu Gly Val Pro  
Glu Arg Ala Ile Val Phe Ser Glu Asp Asn Pro Ser Pro Leu Val Ile Phe Asp Lys Asp Leu Thr Asp Ile Ser Ala  
Gln Leu Thr Ala Ala Leu Gln Ala Gly Gly Leu Asp Leu Pro Thr Ala Thr Leu Leu Gly Thr Gln Phe Gly Gln  
Cys Arg Gln Ala Thr Glu Asn Asp Leu Val Leu Leu Thr Ala Lys Ser Val Ile Gly Lys Val Asn Glu Leu His Arg  
Asp Ala Leu Val Asn Met Gly Val Pro Leu Leu Asp Ala Thr Gln Leu Ser Ile Glu Gly Ile Thr Tyr Pro Ile Glu  
Asp Gln Trp Ile Leu Thr Glu Asn Glu Thr Gly His Val Arg Asn Val Thr Ala Lys Leu Asn Gly Phe Ile Thr Ala  
Ile Ala Asp Ala Asn Asp Asn Val Val Val Ala Asp Met Ala Ala Val Met Gln Asp Met Ser Asn Gly Leu Ile Val  
Glu Asp Gly Ser Ile Tyr Thr Ala Asp Tyr Phe Asn Gly Lys Asn Leu Asp Glu Leu Ser Phe Gly Leu Asp Gly Val  
His Pro Thr Pro Arg Gly Tyr Ala Ile Ile Ala Asn Glu Phe Ile His Val Ile Glu Lys Asn Phe Gly Ala Lys Leu Pro  
Lys Leu Ile Pro Ala Gln Tyr



SEQ ID NO:13 BD423

### Figure 11A

[illegible]

**Figure 11B**

SEQ ID NO:14

BD423

Met Gly Ala Phe Glu Ala Thr Ser Ala Gln Gly Asp Val Ala Ser Arg Asp Glu Leu Ala Glu Ala Ala Ser Asp  
 Glu Ala Val Ala Gln Arg Glu Met Leu Thr Ala Phe Leu Gly Met Cys Asp Thr Glu Glu Ile Ala Pro Ser Ala Gly  
 Leu Thr Val Ser Glu His Arg Val Ala Ser Gln Pro Asp Gly Asn Lys Ile Asn Ile Arg Phe Ile Arg Pro Glu Gly  
 Asp Asp Val Leu Pro Cys Val Tyr Tyr Ile His Gly Gly Met Ala Ser Met Ser Cys Tyr Asp Gly Asn Tyr Arg  
 Ala Trp Gly Arg Ile Ile Ala Ala Gln Gly Val Ala Val Ala Met Val Asp Phe Arg Asn Ala Leu Thr Pro Ser Ser  
 Ala Glu Glu Val Ala Pro Phe Pro Ala Gly Leu Asn Asp Cys Val Ser Gly Leu Lys Trp Val Ala Ala Asn Ala Ala  
 Asp Leu Arg Ile Asp Pro Ala Arg Ile Val Val Ala Gly Glu Ser Gly Gly Gly Asn Leu Thr Leu Ala Thr Gly Leu  
 Lys Leu Lys Arg Asp Gly Asp Leu Gly Leu Ile Lys Gly Leu Tyr Ala Leu Cys Pro Tyr Ile Ala Gly Glu Trp Pro  
 Leu Pro Gln Asn Pro Ser Ser Thr Glu Asn Glu Gly Ile Leu Leu His Leu His Asn Asn Arg Gly Arg Met Thr Tyr  
 Gly Ile Glu Glu Phe Glu Ala Arg Asn Pro Leu Ala Trp Pro Gly Phe Ala Thr Glu Asp Asp Val Ala Gly Leu Val  
 Pro Thr Ile Ile Ser Val Asn Glu Cys Asp Pro Leu Arg Asp Glu Gly Val Gly Phe Tyr Arg Leu Leu Arg Ala  
 Gly Val Pro Thr Arg Cys Arg Gln Val Met Gly His His Pro Arg His Arg Asp Leu Pro Asp Ala Val Pro Arg Arg  
 Glu Pro Gly His Arg Gly Gln His Arg Arg Leu Arg Pro Gln Arg Arg Val Leu Arg Ser Arg Arg Ala Thr Ala  
 Val Pro Arg Val Gly Ala Arg Arg Thr Gln Arg Ala Gly Ser Thr Arg